Specificity and usefulness of an IgE immunosorbent agglutination assay for toxoplasmosis

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Abstract

Aims—To develop an immunosorbent agglutination assay for the detection of *Toxoplasma gondii* IgE antibodies (IgE-ISAGA); to assess its specificity; and to determine the role of specific IgE in the diagnosis of current toxoplasma infection.

Methods—Rabbit antihuman IgE capture antibody was adsorbed onto microtitre plates and formaldehyde fixed tachyzoites were used to identify specific antibody. Specificity was assessed in 513 serum samples (blood donor, potentially interfering and difficult, elevated and low total IgE and myeloma). Serum samples (n = 108) from 65 patients with documented toxoplasmosis were tested, as were 26 serum samples from nine pregnant women positive for specific IgM and 30 from 20 HIV positive patients.

Results—IgE-ISAGA was highly specific with only three of 513 (0.58%) positive reactions amongst the control groups, one of which (0.19%) was regarded as a false positive. Elevated total IgE did not influence specific IgE results nor did the presence of abnormal immunoglobulin concentrations. Sixty (92.3%) patients with toxoplasma associated lymphadenopathy had specific IgE in one or more samples. Positive or borderline results were obtained in 68 of 77 (88.3%) serum samples taken up to four months after onset and were also detected for up to 11 months in 21 of 31 (67.7%) sera. Of the nine pregnant women with detectable specific IgM, specific IgE was absent in five (12 specimens). Specific IgE was also detected in 10 of 30 (33.3%) serum samples from the 20 HIV positive patients, which was similar to the number with specific IgM. Neither the specific IgE nor IgM tests could distinguish symptomatic from asymptomatic HIV positive patients.

Conclusions—IgE-ISAGA is sensitive, specific, and easy to perform. Although results suggest that specific IgE may be less helpful than previously claimed, specific IgE has a useful role in the diagnosis of current toxoplasma infection when used in conjunction with other tests.

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Serological diagnosis of current toxoplasma infection usually relies on the demonstration of specific IgM or fourfold increases in IgG antibody titres. However, these techniques have limited use in pregnant and immunocompromised subjects.^{1,2} Infection during pregnancy can have severe consequences for the fetus but the risk is low if infection occurred before conception.³ As specific IgM may persist for several years,¹ it may be difficult to time the infection before conception on a single sample. Diagnosis of congenital toxoplasmosis cannot be based on the presence of IgM as up to 25% of neonates fail to produce IgM.⁴ Therefore, there is a need to develop other serological tests for timing of maternal infection and detection of congenital toxoplasmosis.

In immunocompromised subjects, especially those with AIDS, toxoplasmic encephalitis usually represents reactivation of a latent infection.² Unfortunately, increases in IgG titres occur too infrequently to be of diagnostic value² and IgM titres often do not reach clinically relevant levels.⁵ As the number of immunocompromised patients is likely to increase, more reliable indicators of infection are urgently needed.

Recently, Pinon *et al*⁵ suggested that specific IgE might be a useful diagnostic indicator of current toxoplasma infection. The time taken for the IgE response to antigenic stimuli differs from that of IgM, and may be important in establishing a more accurate time of infection.⁵ Like IgM, IgE does not cross the placenta and so its detection in neonatal serum samples could be indicative of congenital toxoplasmosis. IgE may also be of use for the detection of reactivated infection,⁵ as has been demonstrated in cytomegalovirus (CMV) infection.⁶ To this end, we developed an IgE immunosorbent agglutination assay (IgE-ISAGA) and tested it in several clinical situations to determine whether the use of specific IgE would improve serological diagnosis of current toxoplasma infection.

Methods

Antigen was prepared as described previously.⁷ Peritoneal exudates from cotton rats infected with the RH strain of *Toxoplasma gondii* 66 hours previously were washed three times in phosphate buffered saline (PBS) before being resuspended in a 3% solution of formaldehyde in PBS. After being incubated

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for 16 hours at 4°C, the antigen was again washed three times in PBS before being resuspended in BABS (borate albumin buffered saline) buffer (pH 8.95) (7.012 g NaCl, 3.092 g H₃BO₃, 4 g bovine plasma albumin (fraction V), 24 ml 1N NaOH, made up to 1 litre with sterile distilled water) to produce a stock suspension containing about 250 × 10⁶ tachyzoites/ml.

IgE-ISAGA was performed in 96 well round bottomed microtitre plates (Dynatech Laboratories, Billingshurst, West Sussex). The capture antibody, rabbit antihuman IgE ϵ chain specific (Dakopatts, Copenhagen, Denmark), was diluted to 2.4 µg/ml in 10% concentrated enzyme linked immunosorbent assay (ELISA) coating buffer (Don Whitley Scientific, Shipley, West Yorkshire), and 100 µl was added to each well. The plates were incubated overnight before being aspirated and then dried at 37°C for four hours. Coated plates were stored dry in sealed plastic packets at 4°C.

Samples were diluted 1 in 100 in PBS, and 100 µl was added to each of two wells for the screening test and to three wells for the confirmation test. Positive and negative controls were included on each plate. Plates were sealed and incubated for two hours in a moist chamber at 37°C. After incubation, the plates were washed once with PBS/0.5% Tween-20 and then washed twice in PBS/0.5% Tween-20 for five minutes and twice with PBS alone for another five minutes. Stock suspension of antigen was diluted in BABS buffer (pH 8.95) to 1.25 × 10⁷ tachyzoites/ml and 100 and 150 µl of the diluted antigen were added to the respective wells for the screening test, and 100, 150 and 200 µl for the confirmation test. The plates were then re-sealed, returned to the moist chamber, and incubated overnight at 37°C. In a negative reaction no agglutination occurs and antigen sediments to form a discrete button. This was assigned a score of 0. In a positive reaction antigen is agglutinated by specific IgE and no sedimentation occurs. This was assigned a score of 4. Intermediate results which represented about 25, 50, or 75% agglutination were scored as 1, 2, or 3, respectively. Results were read using the MR-5000 microtitre plate reader (Dynatech Laboratories) and positive results confirmed by the naked eye. Each sample at each concentration of antigen was scored with respect to positive and negative controls tested with the same amount of antigen. Results of the assays performed in duplicate or triplicate were then added to produce a final matrix value with a maximum score of 8 for the screening test and 12 for the confirmation test.

The threshold for the IgE-ISAGA was determined using dilution curves. A sample positive for toxoplasma specific IgE was serially diluted in a negative sample. This produced 10 dilutions from 1 in 2 to 1 in 1024 which were then tested in triplicate with the positive and negative samples in both the screening and confirmation tests.

Mean ISAGA matrices from each sample were used to calculate best fit curves, which was performed by computer using Mulicall (Pharmacia-Wallac, Milton Keynes, UK). The rationale for using a "turning point" (c) is that it is an objective assessment dependent on four characteristics of the curve used to define a sigmoidal plot of the ISAGA matrix reading (y) against the log₂ value of the sample dilution (x), according to the formula:

$$y = D + (A - D) / [1 + (x/c)^B]^M$$

where A and D are the upper and lower matrix values (8 or 12 and 0, respectively), B is a slope factor, which depends on the gradient of the middle section of the curve, and M is a symmetry factor for the curve.

Specificity was assessed by measuring toxoplasma specific IgE in 513 samples. The control group comprised 150 serum samples from blood transfusion donations. Fifty serum samples from Paul-Bunnell positive patients and elevated rheumatoid factor patients (mean (SD), 387 (276) units, normal range <30 units) and a further 120 "difficult" serum samples were also tested. The difficult serum samples comprised haemolysed (n = 20), lipaemic (n = 20) and icteric samples (n = 20), samples from pregnant women (n = 20), and samples which had been inactivated for one hour at 60°C (n = 40). The effect of elevated total IgE on specific IgE was examined using 73 serum samples (mean (SD) total IgE 580 (376) ku/l, normal range < 25 ku/l). Interference caused by non-saturation of capture antibody and from abnormal concentrations of other immunoglobulins was determined using 30 serum samples with low total IgE titres (<25 ku/l) and 40 from myeloma patients (13 cases with IgG, 16 with IgA, nine with IgM, and two IgG and IgA myelomas).

A further 108 samples from 65 patients (one to six samples per patient) with recent or current toxoplasma infection were also tested. All of these patients had lymphadenopathy; 33 also had other non-specific symptoms. Biopsies had been performed on 14 and were suggestive of toxoplasmosis. All patients had a known duration of illness.

Twenty six serum samples from nine patients suspected of having been infected during pregnancy and 30 serum samples from 20 HIV positive patients were also tested. The HIV patients included 10 with clinical evidence of cerebral toxoplasmosis, five with elevated dye test titres (250–1000 IU), and five asymptomatic patients with dye test titres between 15 and 125 IU.

Specific IgE positive samples used to test the specificity of the IgE test and patients' samples were tested by in-house methods for ELISA IgG⁸ and the biotin avidin IgM (BAM) ELISA-IgM.⁹ Positive BAM ELISA-IgM results were confirmed using Toxnostika ELISA-IgM (Organon Technika, Cambridge), a low sensitivity test used to detect high levels of IgM,¹⁰ and total specific antibody was measured using a modified dye test.¹¹ Specific IgM was measured in samples not confirmed as IgM positive by ELISA and

Figure 1 Threshold curves for IgE-ISAGA screening and confirmation tests. The dashed lines represent the threshold—that is, the turning points 3.05 and 2.61 for the screening and confirmation tests, respectively.

Figure 2 Timecourse of specific IgE in toxoplasmosis: Results (●) of 108 serum samples from 65 patients with recorded time of symptom onset. Mean result (○) of all serum samples taken at 15 day intervals. The dashed line represents the best fit of the mean results.

Table 1 Distribution of matrix values in the IgE-ISAGA screening test

Serum samples	Number of samples	IgE-ISAGA matrix							
		Negative			Positive				
		0	1	2	3	4	5	6	7
Control (BT)	150	140	7	2			1		
Paul-Bunnell positive	50	40	8	2*					
RF positive	50	43	7						
Haemolysed	20	14	6						
Icteric	20	14	6						
Lipaemic	20	20							
Pregnant	20	20							
Inactivated	40	33	6	1					
Elevated total IgE	73	63	8	1			1		
Low total IgE	30	24	3	2	1				
Myeloma	40	30	4	5	1				
Total	513	441	55	13	2		2		
(%)	(100)	(85.9)	(10.7)	(2.5)	(0.4)		(0.4)		

* Poor antigen sedimentation in one sample, which was repeated in the confirmation test. BT = blood transfusion; RF = rheumatoid factor.

in the HIV positive group using the more sensitive Toxo-ISAGA IgM kit (Bio Merieux, Marcy-l'Etoile, France).

Results

Antigen was fixed using 3% formaldehyde in PBS. Lower concentrations of formaldehyde produced an antigen which failed to sediment satisfactorily; higher concentrations reduced sensitivity.

The dilution curves for both the screening and confirmation IgE-ISAGA were sigmoidal (fig 1). In addition to the best fit curve the computer also calculated its turning point. For both curves, this occurred at a matrix value of approximately 3 (3.05 and 2.61 for the screening and confirmation tests, respectively). This value was set as the threshold; matrix values of 3 or 4 in the confirmation test were regarded as equivocal and 5 or above as positive.

Of the 513 specimens tested, only four (0.79%) had matrix values $\geq 3/8$ and thus warranted further testing (table 1). One specimen (Paul-Bunnell positive) was retested because of poor antigen sedimentation. Two of the serum samples (low total IgE and myeloma) when repeated became negative. The control group serum remained borderline positive and the elevated total IgE serum was positive. On the basis of other serological results, however, these serum samples probably originated from patients with recent infections. The Paul-Bunnell serum when repeated gave a mid-range positive value (six of 12) but other serology was not suggestive of recent infection (ELISA IgM negative; dye test 15 IU) and therefore was probably a non-specific reaction. This specimen produced quite a diffuse button unlike the sharp buttons usually seen and with experience, it may be possible to identify non-specific reactions.

The remaining 508 serum samples were specific IgE negative (table 1). Matrix values of those samples with elevated total IgE titres had a similar distribution to other groups tested. In the sample which was IgE-ISAGA positive the total IgE concentration was only 311 ku/l.

Specific IgE was demonstrated in 75 of 108 (69.4%) specimens from 65 patients with toxoplasmosis of documented onset. Borderline reactions (matrix value 3–4/12) occurred in a further 14 (12.9%) specimens. All specimens except two were positive or equivocal by the BAM ELISA-IgM screening test, but 19 of these were not confirmed by Toxonostika ELISA-IgM. Of the latter, Toxo-ISAGA IgM was positive in 14, negative in two and borderline in three. Elevated specific IgE titres were present within 10 days of symptom onset, peak concentrations were reached after two to three months, and IgE was still detectable up to 11.3 months later (fig 2). Seventy seven (71.3%) specimens were taken within four months of symptom onset and specific IgE was detected (positive or borderline) in 68 (88.3%). The remaining 31 (28.7%) specimens were taken up to 11

Table 2 Serological results of patients with samples taken over four months after symptom onset

Patient No.	Duration (months)	ELISA		Dye test (IU)	ISAGA	
		IgG	IgM		IgM	IgE*
1	3.1	+	+	125	ND	7
	3.3	+	+	500	ND	7
	4.9	+	—	250	+	1
	6.4	+	—	250	+	0
2	1.6	+	+	500	ND	7
	3.8	+	+	4000	ND	10
	5.2	+	+	2000	ND	10
	9.1	+	+	1000	ND	12
3	1.5	+	+	2000	ND	6
	2.1	+	+	2000	ND	8
	6.1	+	—	2000	+	6
	10.9	+	—	2000	+	4
4	11.3	+	—	500	—	3
	6.7	+	+	1000	ND	9
	7.4	+	+	500	ND	9
	1.2	+	+	1000	ND	11
5	4.6	+	+	1000	ND	9
	4.6	+	+	250	ND	8
	5.5	+	+	1000	ND	2
	5.9	+	+	250	ND	0
6	1.8	+	+	1000	ND	7
	5.0	+	—	1000	+	2
	10.5	+	—	250	+	2
	7.1	+	+	2000	ND	6
7	2.4	+	+	>1000	ND	11
	5.4	+	+	1000	ND	9
	6.8	+	+	250	ND	9
	1.5	+	—	1000	+	6
8	2.5	+	—	2000	+	6
	12.2	+	—	500	+	2
	4.6	+	+	2000	ND	9
	6.8	+	+	250	ND	6
9	4.8	+	+	500	ND	4
	2.8	+	+	500	ND	8
	3.7	+	+	1000	ND	6
	10.8	+	—	250	±	2
10	4.6	+	+	500	ND	7
	6.1	+	+	1000	ND	5
	7.0	+	+	1000	ND	4
	8.1	+	+	1000	ND	2
11	2.7	+	+	1000	ND	10
	7.2	+	—	2000	+	4
	7.1	+	+	500	ND	7
	8.4	+	—	500	+	2
12	6.0	+	+	1000	ND	12

± equivocal; ND = not done.

* IgE-ISAGA maximum 12/12. Results in bold indicate samples taken four months after symptom onset.

months after onset, but 21 (67.7%) still contained specific IgE (fig 2). In samples taken after four months demonstration of specific IgE was usually associated with detection of IgM by ELISA (table 2). However, IgE-ISAGA negative results were found in three of nine samples confirmed as positive by Toxonostika ELISA-IgM. Similarly, IgE negative results were also observed in six of 12 samples which were borderline or positive by Toxo-ISAGA IgM.

Specific IgE was demonstrated in 13 of 26 (50.0%) serum samples taken from nine women suspected of having been infected during pregnancy (table 3). IgE-ISAGA confirmed maternal infection in patients 1-4 and there was evidence of fetal infection in two. In the remaining five patients IgE-ISAGA provided better evidence for excluding infection during pregnancy than either the Toxonostika ELISA-IgM (four of five positive) or Toxo-ISAGA IgM (three of three positive). Patient 4 was positive by IgE-ISAGA and Toxonostika ELISA-IgM in the first specimen of a second pregnancy, at 11 weeks. In subsequent samples, however, IgE-ISAGA was suggestive of infection prior to conception. No samples from any of the babies were available for testing by IgE-ISAGA.

Positive results were obtained in 10 of 30 (33.3%) samples from the 20 HIV positive patients, the same seropositive rate as found by Toxo-ISAGA IgM (table 4). Specific IgE or IgM was detected in fewer patients (three of 10) with evidence of cerebral toxoplasmosis than in asymptomatic patients (four of 10).

Discussion

Elevated specific IgE antibody titres may be indicative of active infection, primary or reactivated, and may be a more useful diagnostic

Table 3 Infection in pregnancy diagnosed by standard serology and IgE-ISAGA

Patient No.	Gestational age (weeks)	Dye Test IU	E-M	I-M*	Infection during pregnancy†	Outcome	I-E*
1	35	500	+	ND	Yes	CT	9
	37	1000	+	ND			9
	39	2000	+	ND			9
	delivery + 5 weeks	> 4000	+	ND			8
2	19	2000	+	ND	Yes	TOP +	10
	8	250	+	ND			9
	10	4000	+	ND			9
	12	4000	+	ND			8
3	30	250	+	ND	Yes	N	4
	31	125	+	ND			6
	38	125	+	ND			6
	11	125	+	ND			5
4	14	125	+	ND	No	N	3
	18	250	+	ND			2
	20	65	+	ND			0
	27	125	+	ND			1
5	NS	500	+	ND	?	NS	2
	10	65	+	ND			1
	15	125	+	ND			1
	22	65	—	12			1
6	22	65	—	12	No	N	1
	39	65	—	12			0
	12	250	+	ND			1
	13	250	—	12			0
7	15	500	—	12	No	N	2
	22	500	—	12			2

* Maximum 12.

† Based on standard test results, excluding IgE.

E-M = Toxonostika ELISA IgM; I-M = Toxo-ISAGA IgM; I-E = IgE-ISAGA; CT = congenital toxoplasmosis, clinically and serologically; TOP+ = termination of pregnancy, parasites demonstrated in tissues; TOPNT = termination of pregnancy, tissues not tested; ND = test not done; N = no evidence of congenital toxoplasmosis; NS = not stated.

Table 4 IgE-ISAGA, Toxostika ELISA-IgM and Toxo-ISAGA IgM results in HIV positive patients

Group	No. of samples (patients)	No. of positive results		
		ELISA-IgM	ISAGA-IgM	ISAGA-IgE
1	19 (10)	2 (1)	6 (3)	6 (3)
2	6 (5)	0 (0)	2 (2)	3 (3)
3	5 (5)	0 (0)	2 (2)	1 (1)
Total	30 (20)	2 (1)	10 (7)	10 (7)

Group 1, clinical evidence of cerebral toxoplasmosis; group 2, no clinical evidence of cerebral toxoplasmosis, elevated dye test titre; group 3, asymptomatic patients.

indicator of recent infection than IgM.^{5,6} Although ISAGA appears to be more sensitive than ELISA, ISAGA is subjective as its interpretation depends on the point at which the threshold is set. In the method described here fitting a computer generated curve provided a non-arbitrary way of determining this value. The success of this approach was shown by the very low false positive rate achieved.

Other workers have rarely considered the affect of total IgE on specific IgE. Bessieres-Cathala¹² found that serum samples from patients with toxoplasmosis showed an inconsistent increase in total IgE and specific IgE persisted in latent infections. In this study, however, IgE was measured by a direct ELISA using a primarily cytoplasmic antigen. Specific IgE reactions may have been masked by specific IgG and IgM antibodies⁵; the inconsistent results may also be explained by the toxoplasma strain, individual patient response, infective dose, or route of infection.¹³ Some parasitic infections cause an increase in total IgE concentrations, although in toxoplasmosis this does not appear to be important.¹⁴ The latter study, however, was carried out in Brazil in an area where parasitic infection is endemic and the normal range of total IgE was 33–915 IU/ml. Our results were based on normal serum samples with total IgE titres below 50 IU/ml, yet total IgE did not affect specific toxoplasma IgE titres. Although one serum with elevated total IgE concentrations contained specific toxoplasma IgE, other data suggested that this was probably a recent infection.

According to other authors,⁵ specific IgE is suggestive of infection less than four months previously. These results were from a group of 29 pregnant women, only two of whom had lymphadenopathy.⁵ Our results in patients with lymphadenopathy indicate that IgE may persist for up to nine to 11 months if borderline results are included. These discrepancies may be because of the differences in the kinetics of infection between the different patient groups, or because those with persisting symptoms may produce more IgE than asymptomatic patients. Nevertheless, we believe that our results are an accurate representation of the spectrum of IgE response. The use of lymphadenopathy as a marker of the onset of toxoplasma infection has limitations as the incubation period before onset of lymphadenopathy and the time lapse before recognition of symptoms vary. However, the latter are more likely to result in an under-

estimate rather than an overestimate of the duration of infection.

Our assay may also be more sensitive. In previous ISAGA methods a less sensitive antigen may have been obtained due to the enzymatic action of trypsin used to release parasites from sarcoma cells.^{5,15} Our method of production of parasites in cotton rats yields similar numbers of parasites per animal, but eliminated the need for sarcoma cells and hence trypsin digestion. Although difficulties have been described when using antibodies raised in rabbits,^{5,16,17} our assay was both sensitive and specific. The sensitivity of the IgE-ISAGA could limit its use as an indicator of acute infection and a less sensitive, fully quantitative ELISA may be more useful. However, unlike the situation with IgM assays, the IgE capture ELISA has been found to be more sensitive than the IgE-ISAGA.¹⁸ This paradox could be explained by the physical properties of the IgE molecule, which has a lower binding valency than IgM. The IgE molecule may be less efficient at binding the particulate antigens used in ISAGA than the soluble ones used in ELISA, resulting in lower sensitivity. Our IgE-ISAGA could be made artificially less sensitive by arbitrarily raising the threshold, but this would not be justified by our results on specificity.

Persistence of specific IgE in children with congenital toxoplasmosis has been described previously.^{19,20} In 46% of cases persistent IgE expression was followed by the appearance of retinochoroiditis.²⁰ Similar numbers of specific IgE positive results have been observed in children and adults with retinochoroiditis using an ELISA.¹⁸ As very few specimens were tested, the use of specific IgE for confirming ocular toxoplasmosis warrants further study.

Absence of specific IgE in the presence of specific IgM by ELISA was obtained in both lymphadenopathic and pregnant subjects. In the pregnant women this was useful as it excluded infection during pregnancy in four of nine cases, whereas in another four cases positive IgE results confirmed infection. Timing of the specimen in one patient (case 6) was not stated and therefore a negative IgE-ISAGA result in this sample could not exclude infection during pregnancy. Thus, our results indicate that use of the ISAGA technique for the measurement of IgE concentrations may be a better indicator of acute infection than IgM measured by ELISA or ISAGA.

As reactivation of latent infection in immunocompromised patients can have devastating consequences, it is important to diagnose infection. In patients with AIDS the sensitivity of an IgE assay was almost twice that of an IgM assay.²¹ However, IgM was measured by ELISA, which may have operated false negative results. Similarly, in another study⁵ specific IgE was demonstrated by ISAGA in three of seven immunocompromised patients with reactivated infection (four following transplant and three with AIDS), whereas high IgM concentrations were found only in one patient.⁵ These results differ from

those presented here, where the Toxo-ISAGA IgM appeared to be as sensitive an indicator of current infection as the IgE-ISAGA. Nevertheless, demonstration of specific IgE may still be an important addition to the testing repertoire in immunocompromised patients warranting further investigation.

In conclusion, the IgE-ISAGA presented here is easy to use and was found to be both sensitive and specific. Furthermore, it was not affected by abnormal concentrations of total IgE. Among uncomplicated cases of toxoplasmosis, measurement of specific IgE probably has little advantage over detection of specific IgM. However, as specific IgE in most samples is detectable for a shorter period than IgM, it may give a greater indication of current infection. When used in conjunction with other tests, our results suggest that a specific IgE test is useful for the diagnosis of infection during pregnancy and reactivated infections in immunocompromised patients.

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