

Toxocariasis: serological diagnosis by enzyme immunoassay

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SUMMARY An enzyme-immunoassay was developed to measure the concentration of serum antibody specific for the secretory antigens released by migrating toxocaral larvae. This technique was evaluated by testing sera from healthy UK adults, and from patients with and without toxocariasis. In 922 healthy adults, 2.6% were found to have elevated specific antibody levels. Elevated values were observed twice as frequently in males as in females but showed no significant regression with age between 20 and 65 years. Of 62 patients with non-toxocaral helminthic infections, all had anti-toxocaral antibody levels within the range of values observed in healthy controls and had a mean level which was not significantly elevated. All of 13 patients with clinical toxocariasis had enzyme-linked immunosorbent assay (ELISA) antibody levels above the 100th percentiles of both the healthy population and the helminth-infected group and had a significantly high mean value ($P < 0.001$) more than 12 times that of the healthy or infected controls.

The high degree of sensitivity and specificity of the toxocariasis enzyme-immunoassay indicates that this new test should be useful in reference immunodiagnostic applications and in large-scale seroepidemiological surveys.

The confirmation of the diagnosis of toxocariasis depends heavily on immunological tests because parasites may be few in the tissue of those infected and, unless situated in an organ such as the eye, may be difficult or impossible to locate (Woodruff, 1970; Schantz and Glickman, 1978). It was for this reason that in 1964, when work on toxocariasis was begun in this department, a search was made for methods of diagnosis more specific and sensitive than the haemagglutination, flocculation, and complement fixation techniques which until then had been used. Arising from the early work was the development of the toxocariasis skin test in 1964 (Woodruff *et al.*, 1964) followed by the adaptation of the fluorescent antibody test to toxocariasis (Bisseru and Woodruff, 1968) and the subsequent modifications which improved its specificity (Woodruff, 1970). Means whereby further increases in sensitivity and specificity could be obtained have continually been sought. The adaptation by Voller *et al.* (1976a) of the highly sensitive enzyme-

immunoassay procedures for the serological diagnosis of a variety of infectious diseases stimulated this enquiry as to whether the enzyme-linked immunosorbent assay technique could be adapted with advantage for toxocariasis.

Because the more sensitive serological tests require antigens of higher purity and specificity, the recently described larval secretory product (de Savigny, 1975; de Savigny and Tizard, 1977), a toxocaral specific antigen extracted from *in vitro* culture of the parasite, was selected for this work. A microplate enzyme-immunoassay using larval secretory antigen has now proved to be very sensitive and specific and is here described.

Patients and methods

ELISA PROCEDURE

The enzyme-immunoassay method used was the indirect microplate enzyme-linked immunosorbent assay (ELISA) described by Voller *et al.* (1976b). ELISA was adapted to toxocariasis by using *Toxocara canis* second-stage larval secretory antigen (de Savigny, 1975) obtained from *in vitro* culture in

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chemically defined, low molecular weight medium. Secretory antigen was extracted by hollow-fibre ultrafiltration of the culture fluid and was stored at -70°C . For use, antigen was optimally diluted to $0.15\ \mu\text{g}/\text{ml}$ of protein in carbonate buffer, pH 9.6, and, by employing passive adsorption at 4°C for 2 hours, $200\text{-}\mu\text{l}$ volumes of dilute antigen were coated onto the wells of disposable polyvinyl ELISA plates (Cooke, Microtiter 220-29). The plates were washed in phosphate buffered saline containing 0.05% Tween 20 (PBS-Tween), pH 7.4, to remove unadsorbed antigen, and the wells were incubated for 3 hours at room temperature with replicate $200\text{-}\mu\text{l}$ volumes of patients' or reference sera diluted 1:1000 in PBS-Tween. Further washing removed unbound serum components. To assay specifically bound antibody, the wells were incubated overnight at 4°C with $200\ \mu\text{l}$ of alkaline phosphatase-labelled anti-human immunoglobulin. After washing, $200\ \mu\text{l}$ of enzyme substrate, *p*-nitrophenyl phosphate (1 mg/ml) were added at 20°C . The enzyme hydrolysis of substrate was monitored. After the appropriate interval, as determined by the rate of reaction of a reference standard serum, hydrolysis was stopped by the addition of $50\ \mu\text{l}$ of 3M sodium hydroxide. Results (ELISA values) were determined photometrically at 405 nm and expressed in extinction units (E_{405}).

SUBJECTS

Toxocara ELISA was evaluated by testing sera from three groups of subjects: (1) healthy adults; (2) patients with non-toxocaral helminthiasis; and (3) patients with clinical toxocariasis.

Sera from 922 healthy adults were obtained from blood donors native to the UK attending blood donor clinics in south-east England. Donors had a mean age of 34.5 (range 19-65) years and a male:female ratio of 1.01:1.

Sera from 62 subjects with non-toxocaral helmin-

thiasis were obtained from European patients examined at the Hospital for Tropical Diseases, London, and diagnosed as having a single parasitologically diagnosed helminthic infection and whose sera contained demonstrable antibodies to their respective parasites. Sera from patients with clinical toxocariasis were obtained from 10 boys and three girls, aged 2 to 5 years, with a syndrome of prolonged hypereosinophilia ($> 20\%$) associated with at least two of the following features: hepatomegaly, pulmonary infiltration, pneumonitis, fever, geophagia. All 13 patients were reactive to toxocaral somatic antigen by immunofluorescence (Bisseru and Woodruff, 1968) and to secretory antigens by both passive haemagglutination and soluble antigen fluorescent antibody (SAFA) (de Savigny and Tizard, 1977) and by microprecipitation on live larvae (Stevenson and Jacobs, 1977).

Results

PRECISION OF ASSAY

The precision of the *Toxocara* ELISA test was assessed during replicate tests on the 997 control and patients' sera by day-to-day measurements of a quality-control serum included with every 12 specimens. The coefficient of variation thus obtained was 7.7%.

TOXOCARA ELISA VALUES IN HEALTHY ADULTS

The distribution and limits of *Toxocara* ELISA values in healthy UK adults were established from a survey of 922 blood donors (Fig. 1). The distribution appears log-normal, has a median ELISA value of 0.17, and has a small subpopulation (confirmed by probability plot) occurring at the higher ELISA values. Based on the probability plot of the distribution, the minimum elevated ELISA value was selected as 0.50 and is approximately three times

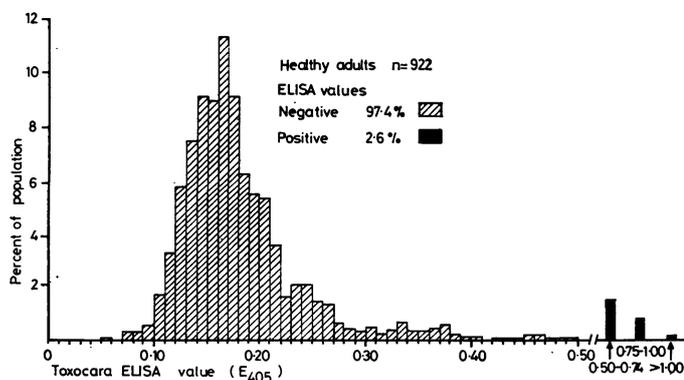


Fig. 1 Distribution of *Toxocara* ELISA values in the healthy adult population ($n = 922$; $M:F = 1.01:1$) sampled from blood donors in south-east England; 2.6% have elevated ELISA values.

the median 'normal' value. Elevated ELISA values were disparate from those of the main population and ranged from 0.50 to 1.35 (3 to 8 times the median-negative) and included 2.6% of healthy adults. Of the 24 blood donors with elevated ELISA values the male:female ratio was 2:1. Although the median ELISA values for both sexes were identical, the change of ratio from 1.01:1 in the total population to 2:1 in the group with elevated values was tested and found to be significant (χ^2 , $P < 0.05$; Wilcoxon rank sum test, $P < 0.01$). Within the group with high ELISA values, males, as well as occurring more frequently, also tended to have significantly higher values (mean 0.82) compared with females (mean 0.60). The significance of the sex-related differences of high ELISA values was confirmed by the method of Cherian and Hill (1978), which corrects for the inherent precision of the assay.

Regression analysis of ELISA value with age between 19 and 65 years revealed no significant association ($P > 0.10$).

TOXOCARA ELISA VALUES IN OTHER HELMINTHIC INFECTIONS

The specificity of the *Toxocara* ELISA test was assessed using sera from 62 Europeans with single parasitologically proved and serologically positive helminthic infections (Fig. 2). The ELISA values of all were within the range found for healthy

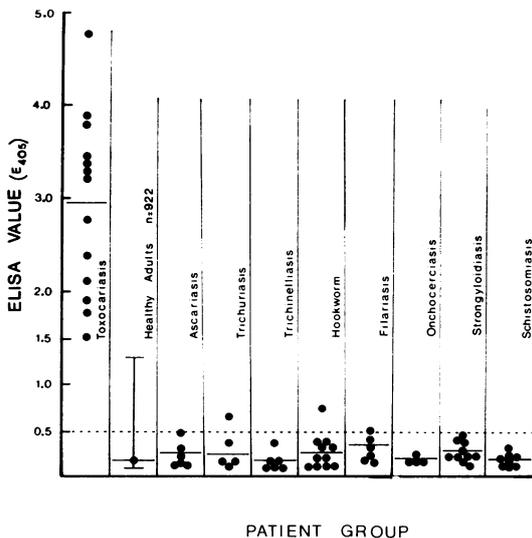


Fig. 2 Comparison of *Toxocara* ELISA values obtained in healthy adults with those obtained in patients with clinical toxocariasis or other helminthic infections. Values above the dashed line are positive. (Vertical line indicates range; horizontal lines indicate means).

controls, and there was no significant difference in mean ELISA value (t test, $P > 0.10$) or in the proportion with elevated values (χ^2 , $P > 0.50$).

TOXOCARA ELISA VALUES IN CLINICAL TOXOCARIASIS

Sensitivity was evaluated by testing sera from 13 cases of clinical toxocariasis (Fig. 2). All had *toxocara* ELISA values above the upper limit observed in the healthy controls. ELISA values ranged from 1.55 to 4.72 (9 to 27 times higher than the median-negative value) with a mean of 2.92, significantly higher than that of both the healthy and helminth-infected controls ($P < 0.001$). To relate this sensitivity in terms of titration endpoints, a positive serum and a negative serum (ELISA values 3.70 and 0.17, respectively) were titrated in 10-fold dilutions (Fig. 3) using $E_{405} = 0.50$ as the titration endpoint. The titres of the positive and negative sera were 1:1 000 000 and less than 1:100, respectively, a dilution difference of approximately 10^4 .

Discussion

The comparison of *Toxocara* ELISA values in healthy adults and in patients with toxocariasis indicates that the microplate-ELISA test using toxocaral secretory antigen is highly sensitive in the detection of *Toxocara* antibody. The absence of

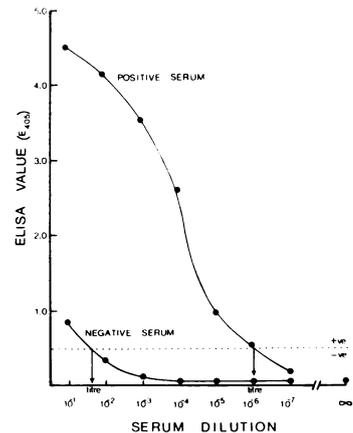


Fig. 3 Sensitivity of *Toxocara* ELISA is demonstrated by titration of a positive and a negative serum. The titre end-point is determined from the highest serum dilution with an ELISA value > 0.50 . In the example shown, the positive titre is 1:1 000 000 while the negative titre is $< 1:100$. To avoid the impracticality of such titrations all sera are tested at a single serum dilution (10^{-3}) and results are recorded as ELISA values (E_{405}).

reactions with sera from patients with other helminthic infections demonstrates a high degree of specificity of the test for toxocariasis.

The quantitative nature of the *Toxocara* ELISA test allows the following general conclusions: *Toxocara* ELISA values less than 0.50 suggest the absence of significant toxocaral infection and are found in 97.4% of the healthy adult population. Values between 0.50 and 1.50 may be expected in 2.6% of the healthy UK population and probably indicate a relatively low level of circulating *Toxocara* antibody such as may be expected in light current infections or after past infections. This figure is consistent with the findings of Woodruff *et al.* (1966), who reported 2.1% of the healthy UK population reactive in intradermal tests for toxocariasis. *Toxocara* ELISA values greater than 1.50 indicate significant levels of specific antibody and are associated with active or recent clinical toxocariasis. Such values do not occur in the healthy population. Although patients with strong clinical and serological evidence of toxocariasis have very high ELISA values (greater than 1.50), it should be expected that patients with light but important infections may have lower positive values between 0.50 and 1.50. For example, of patients with ophthalmic toxocariasis, six were tested and ELISA values ranged from 0.70 to 2.25 (mean 1.45).

It may be questioned whether the 2.6% of healthy adults found to have low levels of circulating *Toxocara* antibody is a reflection of the incidence of recent light infections occurring in adults or is evidence of the prevalence of long-past toxocariasis acquired during childhood. Antibody responses to parasite secretory antigens are expected to be better indicators of active or recent infection than are responses to somatic antigens. However, the level of antibody response to secretory antigen, during active but light infections, tends to be very high (de Savigny and Tizard, 1977). It is not known for how long *Toxocara* larvae persist and continue to migrate in tissues during natural infections in man. That the 2.6% prevalence of low levels of antibody is a result of childhood exposure gains indirect support from the lack of increasing ELISA values during adult life and from the adult male: female ratio of 2:1, which is similar to that observed in clinical toxocariasis occurring in childhood (Snyder, 1961; Huntley *et al.*, 1965).

The high sensitivity of enzyme-immunoassay systems allows the use of small quantities of antigen. It is estimated that the *Toxocara* ELISA test uses approximately 10 ng of antigen adsorbed to each test well. Hence it is possible to perform 60 000 tests with the antigen extracted from one month's culture of 10⁶ larvae. The lack of significant cross-reactivity

of this antigen with other helminths means that adsorption of test sera with helminth antigens is not required. This further suggests that the test may be useful not only in temperate regions but also in the tropics where multiple helminthic infections complicate the interpretation of less specific serological tests.

This work reports the first use of parasite secretory antigen in an enzyme-immunoassay system. The use of highly specific antigen in such a sensitive serological system has made possible a quantitative test with high resolving power. Although sera are tested at a single dilution (requiring only 10 μ l of serum or finger-prick blood), test results are quantifiable on a numerical scale, ranging from 0.05 to 5.0. Serological results in this form are more suitable than titres for seroepidemiological studies where samples from different populations are to be compared (Woodruff *et al.*, in press).

The main disadvantage of the *Toxocara* ELISA test as here described is that it is not practically suitable for small-scale routine diagnostic applications. However, the small amounts of antigen required and the comparatively large numbers of tests that can be performed in a given time render it likely to be more valuable for epidemiological surveys and for central reference diagnosis.

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References

- Bisseru, B., and Woodruff, A. W. (1968). The detection of circulating antibody in human *Toxocara* infections using the indirect fluorescent antibody test. *Journal of Clinical Pathology*, **21**, 449-455.
- Cherian, A. G., and Hill, J. G. (1978). Percentile estimates of reference values for fourteen chemical constituents in sera of children and adolescents. *American Journal of Clinical Pathology*, **69**, 24-31.
- de Savigny, D. H. (1975). *In vitro* maintenance of *Toxocara canis* larvae and a simple method for the production of *Toxocara* ES antigen for use in serodiagnostic tests for visceral larva migrans. *Journal of Parasitology*, **61**, 781-782.
- de Savigny, D. H., and Tizard, I. R. (1977). Toxocaral larva migrans: the use of larval secretory antigens in haemagglutination and soluble antigen fluorescent antibody tests. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **71**, 501-507.
- Huntley, C. C., Costas, M. C., and Lyerly, A. (1965). Visceral larva migrans syndrome: Clinical character-

- istics and immunologic studies in 51 patients. *Pediatrics*, **36**, 523-536.
- Schantz, P. M., and Glickman, L. T. (1978). *Toxocara* visceral larva migrans. *New England Journal of Medicine*, **298**, 436-439.
- Snyder, C. H. (1961). Visceral larva migrans. Ten years experience. *Pediatrics*, **28**, 85-91.
- Stevenson, P., and Jacobs, D. E. (1977). *Toxocara* infection in pigs. The use of indirect fluorescent antibody tests and *in vitro* larval precipitate test for detecting specific antibodies. *Journal of Helminthology*, **51**, 149-154.
- Voller, A., Bidwell, D. E., and Bartlett, A. (1976a). Enzyme immunoassays in diagnostic medicine: Theory and practice. *Bulletin of the World Health Organisation*, **53**, 55-65.
- Voller, A., Bidwell, D. E., and Bartlett, A. (1976b). Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In *Manual of Clinical Immunology*, edited by N. R. Rose and H. Friedman, Chapter 69, pp. 506-512. American Society for Microbiology, Washington, D.C.
- Woodruff, A. W. (1970). Toxocariasis. *British Medical Journal*, **3**, 663-669.
- Woodruff, A. W., Bisseru, B., and Bowe, J. C. (1966). Infection with animal helminths as a factor causing poliomyelitis and epilepsy. *British Medical Journal*, **1**, 1576-1579.
- Woodruff, A. W., Jacobs, D. E., and de Savigny, D. H. (1978). A study of toxocaral infection in dog breeders. *British Medical Journal*, (In press).
- Woodruff, A. W., Thacker, C. K., and Shah, A. I. (1964). Infection with animal helminths. *British Medical Journal*, **1**, 1001-1005.
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