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Visceral Larva Migrans—An Immunofluorescent Examination of Rabbit and Human Sera for Antibodies to the ES Antigens of the Second Stage Larvae of *Toxocara canis*, *Toxocara cati* and *Toxascaris leonina* (Nematoda)

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Summary. Precipitates were demonstrated *in vitro* at the orifices of the second stage larvae of *Toxocara canis* and *T. cati* when placed in sera derived from rabbits infected with these nematodes. Cross-reactions occurred between these two species.

Furthermore, these precipitates occurring at the oral, excretory pore and the anal orifices of these larvae were shown to be of a specific antibody nature by the use of the direct fluorescent antibody (Coons) staining technique.

The second stage larvae of *Toxascaris leonina* did not react in this way when examined in the above-mentioned experimental system, or in sera or globulins derived from rabbits infected with *T. leonina*.

Human sera, taken from clinically suspect cases of visceral larva migrans, were examined in this manner (q.v.). Comparable results were obtained, and it was possible to determine whether fluorescent antibodies were present, and to use this information as an aid to the clinical diagnosis of this disease.

The significance of these findings in relation to the aetiology, pathogenesis and immuno-diagnosis of visceral larva migrans is discussed.

INTRODUCTION

The parasitological problem of visceral larva migrans (VLM) has been discussed by Sprent (1963a) and a clinical assessment in children of this world-wide parasitic zoonosis has been made by Beaver (1962). It seems likely that the species of nematodes involved in the aetiology of this disease would include *Toxocara canis*, *T. cati* and *Toxascaris leonina*, since epidemiologically it is possible for children to ingest the infective ova of these common canine and feline ascarids. *Toxocara canis* larvae have been identified histologically in some of these cases, but the role of the other two species remains uncertain (Beaver, 1962).

Furthermore, the clinical diagnosis of the condition presents some difficulties. Thus, a reliable aid to diagnosis, such as the detection of species-specific antibodies to these invading larvae, would be most useful. Richards, Olson and Box (1962), using a sealed hanging drop technique, carried out a survey for the detection of the presence or absence of precipitins to *T. canis* in sera from suspect children.

Excretory and secretory precipitating antigens of artificially hatched second stage larvae were examined *in vitro* with a near-ultraviolet light microscope using fluorescein isothiocyanate-tagged globulins derived from the sera of normal and infected rabbits, and from the sera of suspected human cases of visceral larva migrans.

Second stage larvae were used because both man and the rabbit are paratenic hosts for these particular nematodes and these larvae are unable to develop any further in such hosts. It was felt that if species-specific antigens do exist then they are likely to be of a secretory or excretory origin and to be associated with the larval stage actually causing the disease.

MATERIALS AND METHODS

Charcoal cultures

Ova were removed by macroscopic dissection from the uteri of the females of all three species and kept in moist washed granulated charcoal in Petri dishes. These preparations were stored in moist chambers in the dark to allow embryonation to take place. When embryonation had occurred (after at least 15 days) the ova were pipetted off from the culture, counted and used for infection of the experimental animals and as a source of larvae for *in vitro* tests.

Rabbits

Disease-free rabbits were kept for the production of normal rabbit sera. Others were infected with 5000 embryonated ova in ground-up dry feed on four separate occasions at three weekly intervals. The rabbits infected with *Toxascaris leonina* were given 50,000 ova in all.

Collection of rabbit sera

Rabbits were bled from the marginal vein of the ear, and the sera were stored without preservatives at −20°. Normal sera from controls and pre- and post-infection sera were obtained in this way.

Collection of human sera

Sera from clinically suspect cases of VLM, from patients allergic to nematodes (but without signs or a history of VLM) and from patients suffering from ascariasis and ancylostomiasis were obtained from hospitals in Australia, New Zealand and England.

Hatching of second stage larvae

Fairbairn's method (Fairbairn 1961) was used to hatch second stage larvae from the nematode ova. Hatching was carried out in a Warburg apparatus using a hatching fluid composed of 0·25 M NaCl containing 0·0025 per cent Tween 80 and 0·1 M Na_2SO_3 and pregassed 0·1 M $NaHCO_3$ (with CO_2). The contents of the flasks were gassed for 15 minutes with N_2–CO_2 (95 per cent : 5 per cent) at 39° after which the taps were closed and hatching allowed to continue for a further 165 minutes. The hatched larvae were then separated from unhatched eggs and culture debris by placing the contents of the flask onto a folded Whatman No. 12 filter paper in a Baermann apparatus (Pitts, 1963). The Baermann apparatus was held overnight at a temperature of 40° and freely moving larvae were then collected from the tube at the bottom of the apparatus. This latter technique applied only to *Toxocara* spp., since it was found that *Toxascaris leonina* larvae were unable to penetrate the filter paper.

FITC conjugation of rabbit sera

To 10 ml samples of each serum, 5 ml of acetate buffer and 35 ml of methanol reagent were added at 0° with continuous mixing (Pillemer and Hutchinson, 1945). The mixture

was then centrifuged at 0° at 3000 rev/min for 30 minutes. The precipitated globulins were resuspended in 20 ml phosphate buffered saline (PBS) to which was added 3 ml cool (4°) carbonate-bicarbonate buffer and 2 ml cool (4°) acetone. Protein was estimated by the Biuret method and the concentration of the protein solution adjusted to 10 mg protein/ml. This solution was brought to a pH of about 9·4 by the addition of 1–2 ml sodium bicarbonate. Fluorescein isothiocyanate (FITC) was then added at 0·025 μg/10 mg of globulin suspension. Conjugation was then allowed to take place by agitation with a magnetic stirrer at 0° for 3 hours. The resulting solution was passed down a Sephadex G25 (Pharmacia) column and allowed to dialyse overnight against a 0·005 M Na_2HPO_4 solution.

Column chromatography was carried out at a pH of 8·1 using diethylaminoethyl cellulose (DEAE). A solution composed of Na_2HPO_4 and KH_2PO_4 (22 ml : 78 ml) was prepared. This solution was used at molarities of 0·02, 0·1, 0·2 and 0·4 to produce, by step wise elution, Fractions II, III, IV and V respectively from the DEAE column with the aid of a Uvicord automatic U/V recorder. Fraction I was collected using 0·005 M sodium dihydrogen phosphate. All fractions were then concentrated to approximately original volume using polyethylene glycol (Carbowax 20,000).

FITC conjugation of human sera

The same methods as those used for the rabbit sera (supra) were employed. The volumes of human sera available varied from 1 to 10 ml and the proportions of reagents used were altered so as to maintain the ratios stated for a starting volume of 10 ml serum.

In vitro *preparations*

Larvae plus FITC conjugated globulins or serum were placed in siliconed 3×1 in. paired well-slides and a coverslip placed over each well. The slides were kept in a moist chamber at 28° and observed every 24 hours for 4 days.

Microscopy

An ordinary light microscope was used for the examination of larvae in rabbit serum. The preparations containing larvae in FITC tagged rabbit globulins were examined with a near-ultraviolet Leitz microscope with a dark ground condenser. Photomicrographs were taken on Tri X (black and white) and Ektachrome (colour) Kodak 35 mm film, using an Orthomat automatic camera. The larvae were killed just prior to photography by heating the slide rapidly on a hot bulb for several seconds. This did not affect the fluorescence, but immobilized the larvae thus allowing the exposure time necessary for UV photomicrography.

RESULTS

Table 1 summarizes the experimental results obtained when larvae were placed in various rabbit sera. The oral, excretory and anal orifices of *Toxocara canis* and *T. cati* larvae developed precipitates when placed in their respective sera and cross reactions occurred between them. *Toxascaris leonina* larvae did not form precipitates in any of the sera. These findings were then extended and confirmed when the larvae were examined for fluorescent antigen–antibody complexes (Figs. 1 and 2). Fraction III (0·1 M) was found to contain the fluorescent antibodies specific for the excretory and secretory antigens at the larval

TABLE 1

In vitro EXAMINATION OF THE SECOND STAGE LARVAE OF *Toxocara canis*, *T. cati* AND *Toxascaris leonina* IN NORMAL AND INFECTED RABBIT SERA, AND IN FITC LABELLED GLOBULINS DERIVED FROM THESE NORMAL AND INFECTED RABBIT SERA

Larvae	Sera or FITC globulins							
	Normal rabbit serum	Serum from rabbit infected with *T. canis*	Serum from rabbit infected with *T. cati*	Serum from rabbit infected with *T. leonina*	FITC tagged globulins from normal rabbit serum	FITC tagged globulins from rabbit infected with *T. canis*	FITC tagged globulins from rabbit infected with *T. cati*	FITC tagged globulins from rabbit infected with *T. leonina*
Second stage *T. canis* larvae	−	+	+	−	−	+	+	−
Second stage *T. cati* larvae	−	+	+	−	−	+	+	−
Second stage *T. leonina* larvae	−	−	−	−	−	−	−	−

+ = Presence of precipitates at oral, excretory and anal pores, and in the case of the fluorescent preparations, the presence of green fluorescent precipitates at the oral, excretory and anal pores in addition to blue auto-fluorescence of the intestine.
− = No precipitates visible, and in the case of the fluorescent preparations blue auto-fluorescence of the larval intestine only was observed.

orifices. Near-ultraviolet light fluorescence microscopy revealed the presence of green fluorescent precipitates at the mouth, excretory pore and anus of *Toxocara canis* and *T cati* larvae when they were placed in *in vitro* preparations of globulins derived from sera from either of these infections. The cross-reactions were clearly visible. *T. canis* larvae fluoresced in FITC globulins derived from sera from rabbits infected with *T. cati* and *T. cati* larvae fluoresced in FITC globulins derived from sera from rabbits infected with *T. canis*. There was an absence of these antigen–antibody precipitates in normal rabbit FITC tagged globulins (Fig. 3) and in FITC tagged globulins from the sera of rabbits infected with *Toxascaris leonina*. In all cases bright blue auto-fluorescence of the larval intestine was visible (Figs. 1–6).

Fraction IV did cause the formation of some fluorescent precipitates similar to Fraction III, and, in addition, fluorescent material appeared at random on the surface of the cuticle (Fig. 4). This type of staining was not seen when Fraction III was used. Fractions I, II and V exhibited no activity.

The fluorescent precipitates were seen to be in various stages of formation, and occasionally pieces of fluorescent precipitate were seen to detach from the moving larvae.

Fluorescent precipitates appeared first at the oral opening in 3–24 hours, at the end of which time the excretory pore was also fluorescing. The excretory pore fluoresced more and more strongly, occasionally developing a bright green band of material around the

FIG. 1. Second stage *Toxocara canis* larva in FITC conjugated globulins from the serum of a rabbit infected with *T. canis*. Yellow-green oral, anal and excretory pore precipitates are visible. Blue auto-fluorescence of the gut can be seen. ×200.

FIG. 2. Same as Fig. 1 at high magnification. A virtually identical picture is obtained with *Toxocara canis* larva in FITC tagged globulin derived from the serum of a rabbit infected with *T. cati*. ×500.

FIG. 3. Second stage *Toxocara canis* larva in FITC globulin preparation derived from normal rabbit serum and exhibiting autofluorescence only. No immune fluorescent precipitates are present. ×500.

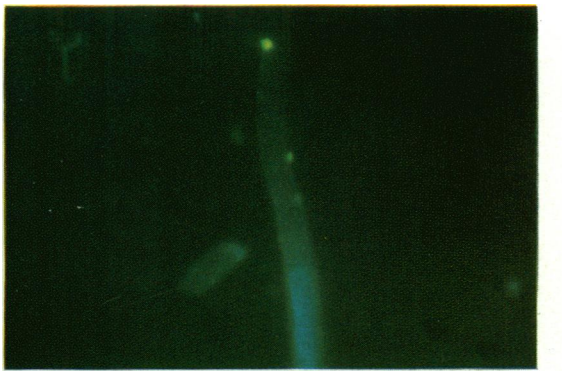
FIG. 4. Second stage *Toxocara canis* larvae showing yellow-green staining at the pores and at random over the cuticle. This type of staining occurs when Fraction IV (0·2 M) of the FITC globulin preparation from *T. canis* infected rabbit serum is used. ×200.

FIG. 5. Second stage *Toxocara canis* larva in FITC conjugated globulins derived from the serum of a child who had contracted VLM. In this particular case an ascaridoid larva had been identified in the enucleated eye of the patient. Oral and excretory pore fluorescent precipitates are visible. A dislodged excretory pore fluorescent precipitate can be observed on the side opposite to the pore. ×500.

FIG. 6. Bands of fluorescent precipitate around the excretory pore of second stage *Toxocara canis* in FITC globulins derived from a histologically supportable case of human VLM. ×500.

Visceral Larva Migrans

1



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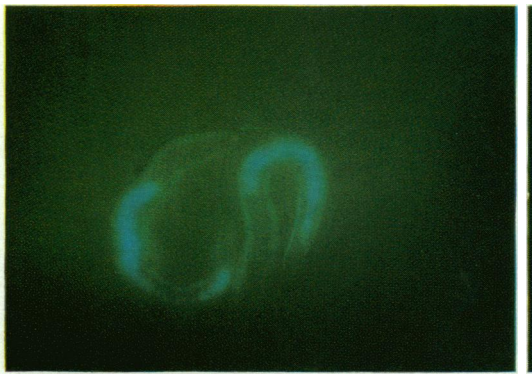

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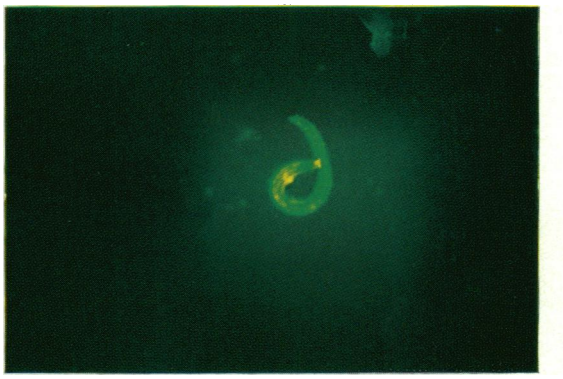

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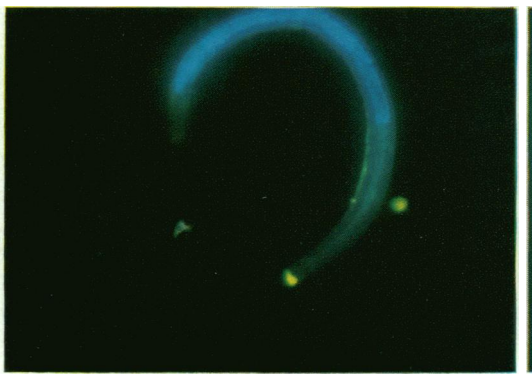

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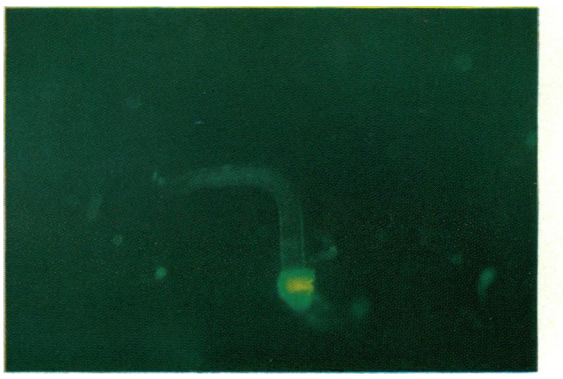

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larva before it broke off. By 48 hours fluorescent precipitates were visible at the anus as well so that all three pores were fluorescing with a bright green colour by this time. The larvae remained alive and continued to fluoresce for 4–5 days after the preparation was set up. These antigen–antibody precipitates continued to fluoresce even after the larvae had died. However, they were easily lost even when the larvae were washed gently. Hence, examination and photography were carried out with the original preparation against a dark green background of FITC tagged globulins.

When the reproducibility of the results had been established for rabbit sera, human sera from patients suspected of having contracted VLM were examined using the same methods.

Again cross-reacting fluorescent precipitates were seen at the orifices of *Toxocara canis* and *T. cati* larvae. Fig. 5 shows fluorescent precipitates at the oral, excretory and anal pores of the second stage larvae of *T. canis* when placed in FITC conjugated globulins derived from a histologically supportable human case of VLM. No fluorescent reaction was observed with *Toxascaris leonina* larvae. Fig. 6 shows a fluorescent aggregate at the excretory pore of a *Toxocara canis* larva. There appear to be bands of precipitated fluorescent material around this pore and, in addition, oral pore fluorescence (the latter is out of focus since it was necessary to focus the camera primarily on the excretory pore).

In all, twenty-eight such sera were examined from Australasian cases of suspected VLM (see Table 2, Survey 1) and ten cases from English hospitals (see Table 2, Survey 2).

TABLE 2

EXAMINATION OF HUMAN SERA FOR *Toxocara* spp. ANTIBODIES BY IMMUNOFLUORESCENCE

Geographical source of sera examined	Total number of suspect cases	Fluorescent antibody present to *Toxocara* spp. ES antigens	No demonstrable fluorescent antibody	Percentage of positives in the sera examined
Australasia (Survey 1)	28	8	20	29
England (Survey 2)	10	3	7	30

In addition, negative immunofluorescent results (i.e. no fluorescent precipitates were visible at the pores of any of the three larval species), were obtained when the sera of three categories of people were examined. Category 1 consisted of sera from healthy people chosen at random and such sera acted as negative controls. Category 2 was composed of sera from parasitology laboratory workers allergic to nematodes (but who had no history of VLM). Category 3 included cases of human ascariasis and ancylostomiasis. These last two categories were examined to detect, respectively, the presence of false positives and cross-reactions; none reacted.

DISCUSSION

Olson (1960) demonstrated the presence of precipitates on *Toxocara canis* larvae in hanging drop preparations with infected sera. Taffs (1961) demonstrated the presence of precipitates at the mouth, excretory pore and anus of third stage *Ascaris suum* larvae when placed in infected pig serum. Further work by Taffs and Voller (1962) using a fluorescent

antibody technique showed that these precipitates around third stage *A. suum* were of an antibody nature.

The results in Table 1 confirm and extend Olsen's findings with *Toxocara canis* and are analogous with those of Taffs *et al.* (1962). In addition, it was possible to demonstrate the presence of cross-reactions between *T. canis* and *T. cati*, and by using FITC tagged globulins, that these precipitates were in fact fluorescent antibodies to excretory–secretory antigens produced by these larvae. Furthermore, in contrast to Olsen's observations, *Toxascaris leonina* did not cross-react in these sera, or in sera and FITC globulins from rabbits infected with *T. leonina*, even though a much higher total number of ova was given. *T. leonina* is not as invasive as *Toxocara* spp. (Sprent, 1956, 1958, 1959) and is taxonomically more closely related to *Ascaris suum* than to *Toxocara* spp. Thus, the sera from rabbits infected with *T. canis* and *T. cati* contain specific precipitating antibody which will react with antigens produced by the living second stage larvae of these nematodes. Such antigens have in the past been referred to as metabolic antigens, but are probably more properly called 'ES' (excretory and secretory) antigens (Thorson, 1963, after Campbell, 1955).

The fluorescent precipitates appeared at the orifices in a fixed time sequence, i.e. first oral, secondly excretory and thirdly anal. After 48 hours all three sites fluoresced, the anal one being the last to appear, presumably after the metabolism had had time to produce waste products at this orifice.

It is pertinent to note that these experimental findings are consistent with Sprent's (1963b) concept of parasitism, and that *Toxocara* spp. are more invasive and in more intimate histological contact with their hosts than *Toxascaris leonina* (the latter being confined to the intestinal wall and associated lymphatic tissues).

Coombs, Pout and Soulsby (1965) have shown, by the mixed antiglobulin reaction, that normal human and rabbit sera possess antibody-like factors (globulins) which combine with the cuticle of live *Turbatrix aceti*. This reaction was also demonstrated with the parasitic nematode *Toxocara canis* (Hogarth-Scott, unpublished). However, such antibodies are distinct from the apparently species-specific precipitating ones produced against the ES antigens of *T. canis* and *T. cati*.

Some evaluation of the results shown in Table 2 is relevant here, although a detailed clinical correlation between cases examined and fluorescent antibody results will be published elsewhere. The usual clinical signs of VLM include hepatomegaly, eosinophilia and hypergammaglobulinaemia, and the VLM syndrome is typically found in children of 15–36 months of age. There is usually a history of pica and of association with young dogs. Most of the children of this age showed all these signs. However, the majority of the sera came from children with endophthalmitis, i.e. older children. It cannot be said that the eye is a predilection site but rather that the disease is more readily detected from eye lesions.

One of the sera giving a positive result in Survey I was from a child whose liver biopsy section, examined histologically, revealed the presence of an ascaridoid larva. Another positive serum was obtained from a child in whose enucleated eye an ascaridoid larva had been identified (Sprent, personal communication). It is rarely possible to identify such a nematode larva as a particular species. Thus, two of the cases in Survey I from which fluorescent antibodies were detected could be supported by direct histological evidence of VLM.

In Survey II, a boy with a very typical history had been bled at an interval of 4 years, and, on both occasions, it was possible to demonstrate the presence of fluorescent anti-

bodies in his serum. This reflects one of the most interesting points of the disease and supports Beaver (1962) who showed that in an infection with *T. canis* all the larvae were not killed despite fibrous encapsulation, and that the larvae occasionally left the capsules, underwent further migration and became re-encapsulated. If such is the case, one would expect persistent intermittent antigenic stimulation of the host by these ES antigens of *Toxocara* spp. larvae. It may also explain why persistent fluctuating eosinophilia is such a marked feature, although the mechanism in such a phenomenon is open to speculation.

Some of the suspect sera giving negative results were taken from very doubtful VLM cases, e.g. an 80-year-old patient. The cases of ascariasis and ancylostomiasis were patent infections, and the allergic sera were obtained from people who became asthmatic and anaphylactoid in the proximity of nematodes.

This paper records a method by which it is possible to demonstrate in rabbit and suspected VLM human sera the presence of precipitating fluorescent antibodies to the ES products of the second stage larvae of *Toxocara* spp. but not to *Toxascaris leonina*.

It is felt that such findings may be of some use in the immunological diagnosis of visceral larva migrans which is compromised at present by aetiological uncertainty and lack of specific antigens for use in a serological test.

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