L-Glutamate binding sites of parasitic nematodes: an association with ivermectin resistance?

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(*Received 13 October 1999; revised 6 December 1999; accepted 6 December 1999*)

SUMMARY

Nematode membrane preparations contain high amounts of low-affinity specific L-glutamate binding sites. The numbers of these sites were increased in 2 isolates, one field-derived and the other laboratory-derived, of ivermectin-resistant *Haemonchus contortus* and a field isolate of ivermectin-resistant *Telodorsagia circumcincta*, when compared to control, drug-sensitive isolates. Specific [^{3}H]ivermectin binding to these membrane preparations showed no differences between ivermectin-sensitive and resistant isolates and the number of ivermectin binding sites was approximately 100-fold less than the number of L-glutamate binding sites. Kinetic analysis of L-glutamate binding suggested the presence of at least 2 classes of binding site. L-Glutamate binding was blocked by ibotenic acid, kynurenic acid and β-hydroxyaspartate, but not by ivermectin, argiopine, kainate, quisqualate or NMDA. Competition assays with ibotenic acid suggested that there were 2 distinct populations of glutamate binding sites and that the site with the lower affinity for ibotenate was upregulated in the ivermectin-resistant nematodes. In the field isolate of resistant *H. contortus* we found no coding changes in the cDNAs encoding glutamate-gated chloride channel subunits HG2, HG3 and HG4, nor were any changes in channel expression detected using subunit-specific antibodies. The low-affinity binding site is unlikely to be associated with the ivermectin receptor in these nematodes.

Key words: ivermectin, glutamate, *Haemonchus contortus*, *Telodorsagia circumcincta*, resistance.

INTRODUCTION

Ivermectin and the other macrocyclic lactone anthelminthics are widely used for the control of parasitic nematodes in animals and humans. Recently, the appearance of ivermectin-resistant nematodes has threatened to limit the effectiveness of this class of compound in veterinary parasitology (Shoop, 1993; Le Jambre *et al*. 1995; Waller *et al*. 1996; Stafford & Coles, 1999) and may, in future, pose a threat to the continued success of programmes to treat onchocerciasis and related human conditions. Any strategy for the management of anthelminthic resistance requires rapid and inexpensive tests for its detection, and the development of such tests in turn requires an understanding of the genetic or biochemical basis of the resistance phenotype.

Genetic studies on field isolates of ivermectin-resistant nematodes have suggested that resistance is dominant, implying a gain-of-function mutation (Le Jambre, 1993; Dobson, Le Jambre & Gill, 1996), though this may not be true for laboratory selected isolates (Gill & Lacey, 1998). Mutations in several genes have been implicated as involved in the development of resistance in *Haemonchus contortus*, including those encoding P-glycoproteins (Xu *et al*. 1998; Sangster *et al*. 1999) and putative components of the L-glutamate-gated chloride channel (Blackhall *et al*. 1998). Kotze (1998) reported that pharyngeal pumping, one of the major targets of ivermectin (Bottjer & Bone, 1985; Geary *et al*. 1993) was less sensitive to the drug in resistant *H. contortus* larvae and Paiement *et al*. (1999*a*) have observed a reduced inhibition of inulin uptake (a measure of pharyngeal pumping) by ivermectin, but not moxidectin, in a single laboratory isolate of ivermectin-resistant *H. contortus*. Rohrer *et al*. (1994) detected no difference in ivermectin-binding sites in membrane preparations of a laboratory-derived ivermectin-resistant *H. contortus* isolate from those of its susceptible parent. A recent report has suggested that a high-abundance, low-affinity L-glutamate binding site is present at higher levels in the ivermectin-resistant isolates (Paiement, Prichard & Ribeiro, 1999*b*). Studies on other isolates of ivermectin-resistant parasitic nematodes have produced conflicting results. Gill *et al*. (1998) reported that the use of

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https://doi.org/10.1017/S0031182099005843 Published online by Cambridge University Press

different selection protocols to produce resistant *H. contortus* induced different resistance phenotypes. A gradual selection protocol using slowly increasing doses of ivermectin did not result in any decrease in sensitivity of larval motility or development. A single-step selection with an ivermectin concentration 10 times the LD_{95} produced a resistance phenotype involving a reduced inhibition of larval motility and development by the drug *in vitro*. The latter phenotype was more similar to the resistance phenotype observed in field isolates of *H. contortus* (Gill *et al*. 1991, 1995; Le Jambre *et al*. 1995). These observations clearly demonstrate the need to confirm any suggested mechanism of resistance observed in laboratory-derived resistant isolates in field isolates. In addition, it has been suggested that the mechanism by which ivermectin treatment results in expulsion of parasites from the host may differ between nematode species (Gill & Lacey, 1998). In particular, *T. circumcincta* adults tended to be expelled from the abomasum of infected sheep more slowly than *H. contortus* after oral treatment with 0·2 mg/kg ivermectin. This suggests that the action of ivermectin on the two species may differ and raises the possibility that they may develop different means of resistance to overcome the anthelminthic.

One common mechanism of pesticide resistance is an alteration in the site of action, and this has been demonstrated for the nicotinic agonist, levamisole (Robertson, Bjorn & Martin, 1999) as well as for the benzimidazoles (Kwa *et al*. 1995). The probable sites of action of ivermectin and related anthelminthics are the L-glutamate-gated chloride channels (Arena *et al*. 1992), which are closely related to vertebrate $GABA_A$ receptors and are therefore likely to exhibit the same heteropentameric structure. Alterations in these channels might be expected to result in changes to L-glutamate binding to membranes of resistant worms. L-glutamate-gated chloride channels have been studied most extensively in *Caenorhabditis elegans*, where 5 genes have been identified that encode subunits of these receptors (Cully *et al*. 1994; Dent, Davis & Avery, 1997; Laughton, Lunt & Wolstenholme, 1997; Vassilatis *et al*. 1997; Horoszok, Sattelle & Wolstenholme, 1999). Two of these genes, *avr-15* and *gbr-2*, are alternatively spliced to produce 2 different subunits (Dent *et al*. 1997; Laughton, Lunt & Wolstenholme, 1997; Vassilatis *et al*. 1997); mutations in the *avr-15* gene contribute to high-level ivermectin resistance in *C. elegans* (Dent *et al*. 1997). Molecular cloning studies on *H. contortus* have identified 3 probable glutamate-gated chloride channel subunit genes (Delany, Laughton & Wolstenholme, 1998; Forrester *et al*. 1999; Jagannathan *et al*. 1999). Two of these genes are clearly orthologous to the *C. elegans* GluCl β and *gbr-2* genes; the *H. contortus gbr-2* gene is also alternatively spliced to yield 2 subunits (Delany *et al*. 1998; Jagannathan *et al*. 1999). The third gene has been designated as encoding a GluCl α-subunit (Forrester *et al*. 1999) and population genetics studies have suggested that alterations in this gene might be associated with ivermectin resistance in *H. contortus* (Blackhall *et al*. 1998).

We have examined the possible involvement of glutamate- and ivermectin-binding proteins in ivermectin resistance in parasitic nematodes. Most of our initial experiments were carried out using the White River II field isolate of *H. contortus* (van Wyk & Malan, 1988). Where significant differences have been found between this isolate and susceptible worms, we have attempted to confirm these differences in an additional isolate of ivermectin-resistant *H. contortus* generated in the Langford laboratory and a resistant field isolate of *T. circumcincta* (Coles, Warner & Best, 1996). We can find no evidence for any change in ivermectin binding between susceptible and resistant worms, but confirm a very large increase in a population of low-affinity L-glutamate binding sites in ivermectin-resistant nematodes.

MATERIALS AND METHODS

Materials

[^{3}H]L-glutamate (specific activity 1·7–2·2TBq mmol^{-1}) was purchased from Amersham-Pharmacia International (Aylesbury, UK). [^{3}H]Ivermectin (specific activity 1·74TBq mmol^{-1}), custom synthesized by Amersham-Pharmacia International, was a kind donation from Pfizer (Kent, UK). Unlabelled L-glutamate, ivermectin (avermectin B1), HEPES, EDTA, leupeptin and pepstatin were from Sigma (Poole, UK).

The IVM-resistant field isolate *H. contortus* was from the White River in South Africa (van Wyk & Malan, 1988); this isolate is also resistant to benzimidazoles. A manuscript detailing the production of the Langford ivermectin resistant isolate of *H. contortus* from the susceptible isolate used in this study is currently in preparation. The ivermectin-resistant field isolate of *T. circumcincta* was isolated from Angora goats in the south west United Kingdom (Coles *et al*. 1996); this isolate is also resistant to levamisole and benzimidazoles.

Nematode larval culture

Worm-free lambs maintained on concrete floored pens were infected with 5000 larvae of either susceptible or ivermectin-resistant *H. contortus* or 10000 larvae of susceptible or resistant *T. circumcincta*. Twenty-one days after infection lambs were fitted with a harness and faeces collected into a bag overnight. Faeces were incubated for at least 14 days at room temperature before L3 larvae were collected by Burmannization. Larvae were purified by cen-

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trifugation over saturated sucrose before being thoroughly washed with tap water and stored at −80 °C. Eggs were collected from faeces by differential sieving and centrifugation through saturated sodium chloride as described previously (Laughton *et al*. 1994).

Binding assays

Crude membrane preps were prepared from L3 larval stages of both ivermectin-sensitive and resistant isolates. Approximately 2 g of frozen larvae were rapidly thawed and washed with cold lysis buffer [50 mM HEPES (pH 7·4) containing 1 mM EDTA, 0·2 mM PMSF, leupeptin (0·5 μg/ml) and pepstatin (0·7 μg/ml)]. EDTA was omitted from the lysis buffer for membrane preparations used in [^{3}H]ivermectin binding assays. Nematodes were resuspended in 7 ml of the lysis buffer along with approximately 20 crushed glass cover-slips followed by about 5 cycles of vortexing for 1 min and cooling for 2 min until most of the worms were broken, as judged by microscopic inspection. The volume of the homogenate was made up to 40 ml with 50 mM HEPES (pH 7·4) and centrifuged at 1000 ***g*** for 10 min (P1 fraction). The supernatant (S1 fraction) was centrifuged again at 28000 ***g*** for 30 min at 4 °C and the pellet (P2 fraction) resuspended in 50 mM HEPES (pH 7·4). The protein concentration was measured using the Bradford method (BioRad Labs) with BSA as standard.

Binding of radio-isotope labelled ligands to P2 membrane preparations was measured using saturation and competition binding studies. Membranes were incubated with [^{3}H]L-glutamate in 50 mM HEPES (pH 7·4) with typically 80 μg of protein in a final assay volume of 100 μl. Non-specific binding was defined by binding in the presence of 3 mM unlabelled L-glutamate. Incubations were at room temperature (~ 22 °C) for 2 h. Reactions were terminated by dilution with 200 μl cold 50 mM HEPES (pH 7·4) followed by rapid filtration (Brandel cell harvester) through type A/B glass fibre filters (Gelman Sciences) which were presoaked in 0·3 % (w/v) polyethyleneimine (PEI). Filters were washed again with 10 × 200 μl of 50 mM HEPES (pH 7·4) at 4 °C. Bound radioactivity was determined by liquid scintillant spectrometry with a counting efficiency of 40–50 %. All experiments were repeated between 3 and 12 times and all values shown are mean ± S.E.M. For the competition studies, P2 membrane preparations were incubated with 2 μM [^{3}H]L-glutamate for 2 h at room temperature with various dilutions of competing ligands. Non-specific binding was determined in the presence of 3 mM unlabelled L-glutamate. Data were fitted to the Hill equation to determine the IC_{50} values for each competitor. Values are the mean of 3 individual experiments done in duplicate.

Kinetic analysis

Association and dissociation studies were performed using 2 μM [^{3}H]L-glutamate. Association rates were determined by incubation of P2 membrane preparations with radioligand at 4 °C for 0·5–240 min before termination by rapid filtration over glass fibre A/B filters. Dissociation rates were determined following pre-incubation of membrane preparations with 2 μM [^{3}H]L-glutamate at room temperature for 120 min (in order to reach equilibrium) followed by 4 °C for 16 h. Dissociation was initiated by the addition of 1 mM cold unlabelled L-glutamate and measured for 1–240 min.

Data analysis

Non-linear regression analysis (Sigma Plot v 4.0, Jandel Scientific) was used to determine the dissociation constant (K_d) and the maximal binding capacity (B_{max}) fitted to a single site ligand binding model. Data were fitted to the equation $y = (B_{max}[^3\text{H-ligand}])/K_d + [^3\text{H-ligand}]$. Each experiment was done with replicates and experiments were repeated 3–12 times. Dissociation (K_{off}) and association (K_{on}) rates were determined from the kinetic binding studies. Dissociation data were fitted to both 1 and 2 site models for comparison. Linear regression analysis was used to estimate the Hill coefficient and the Scatchard analysis of the specific [^{3}H]-ligand binding.

Cloning and localization of glutamate-gated chloride channel subunits

Oligonucleotides corresponding to the 5′ and 3′ ends of the HG2, 3 and 4 cDNAs (Delany *et al*. 1998; Jagannathan *et al*. 1999) were used in RT–PCR reactions on mRNA isolated from the eggs of the White River isolate of *H. contortus*. Immunological localization of the expression of these subunits in adult White River *H. contortus* was carried out as previously described (Delany *et al*. 1998; Jagannathan *et al*. 1999).

RESULTS

Characterization of L-glutamate binding to H. contortus *membranes*

We initially compared the extent of [^{3}H]L-glutamate binding to the different fractions of the membrane preparations of *H. contortus* L3 larvae. This stage was used as it was relatively cheap and straightforward to obtain sufficient material to permit the number of experiments required for the production of good-quality data. Incubation of subcellular fractions with 2 μM [^{3}H]L-glutamate in 50 mM HEPES buffer (pH 7·4) showed that the highest specific binding was in the P2 fraction of the

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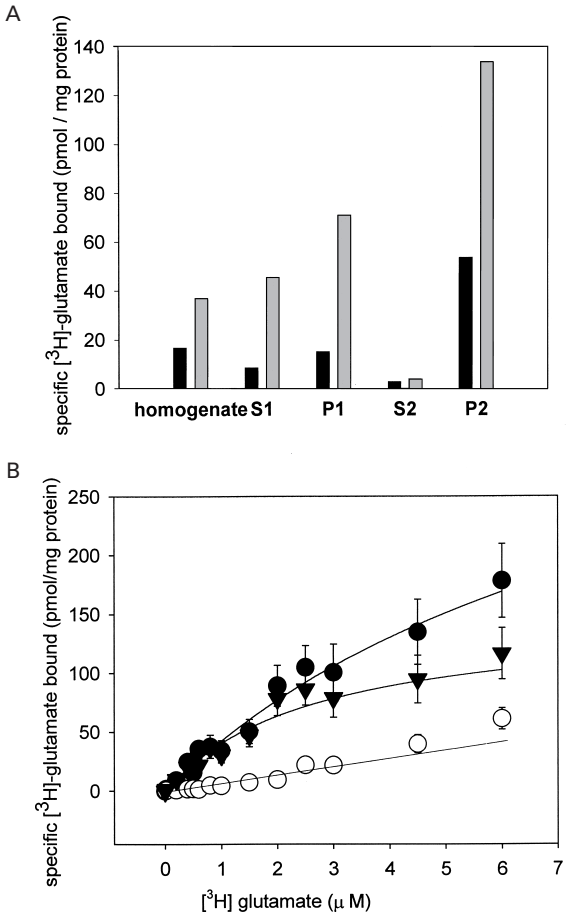

Fig. 1. Characterization of [^{3}H]L-glutamate binding to *Haemonchus contortus* membranes. (A) Specific binding to membrane fractions of ivermectin sensitive (■) and resistant (White River) (▨) prepared as described in the text. (B) Saturation curve of L-glutamate binding to P2 membranes from ivermectin-sensitive *H. contortus*. Specific binding (▼) is calculated as the difference between the binding seen in the presence (○) and absence (●) of a saturating concentration (3 mM) of non-radioactively labelled L-glutamate.

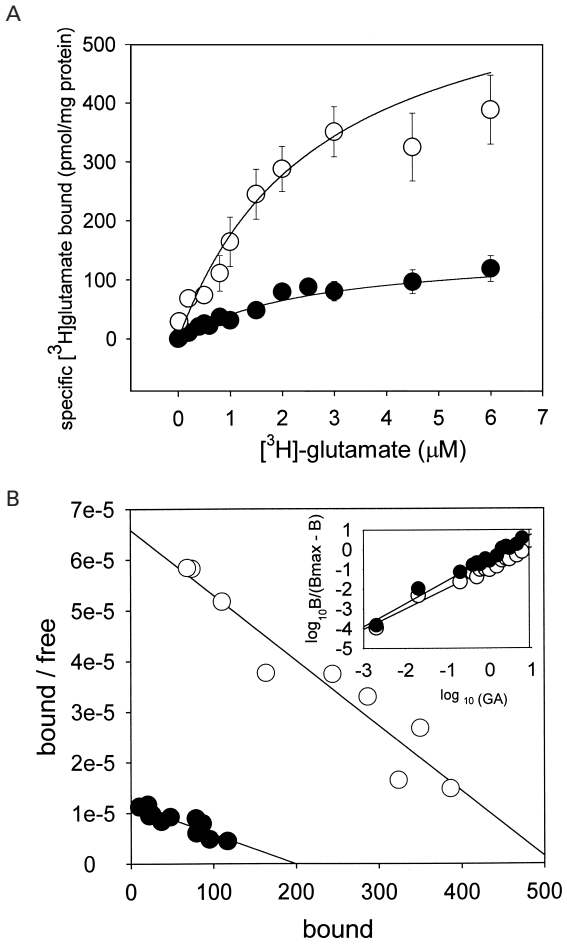

Fig. 2. Equilibrium binding of [^{3}H]L-glutamate to P2 membranes of ivermectin-sensitive (●) and resistant (White River) (○) *Haemonchus contortus*. (A) Saturation curve. (B) Scatchard plot of the data in (A). The inset is the Hill plot.

membrane preparation (Fig. 1). The same result was observed with both ivermectin-susceptible and resistant (White River isolate) L3 larvae. Non-specific binding defined by the presence of 3 mM glutamate was less than 10% of the total in both preparations. The dependence of [^{3}H]L-glutamate binding on protein concentration was analysed under these conditions with an incubation time of 2 h at room temperature. Specific binding increased linearly up to a concentration of 2 mg/ml in both isolates (data not shown). Further studies were therefore performed at a protein concentration of 0·8 mg/ml, since this was in the linear range and yielded sufficient binding for accurate analysis.

Specific binding of [^{3}H]L-glutamate to P2 membrane preparations from ivermectin sensitive *H. contortus* was saturable with low affinity binding to a single site of $K_d = 2{\cdot}64 \pm 1\ \mu$M and a B_{max} of 148·3 + 21·3 pmol/mg protein (Fig. 1B). The Hill coefficient was close to unity ($n_H = 0{\cdot}97$). Specific [^{3}H]L-glutamate binding was approximately 90% of the total binding at a radioligand concentration equal to the K_d. This result suggested the presence of a very large number of low-affinity L-glutamate binding sites in *H. contortus* membranes, though the actual figures derived should be treated with caution as we were unable to achieve true equilibrium conditions in these assays. In particular, the errors associated with the calculation of the K_d were rather large. We attempted to specifically measure a higher-affinity population of L-glutamate binding sites by using a shorter incubation time, 8 min, and a lower temperature (4 °C). Under these conditions Schaeffer *et al.* (1990) observed a less abundant population of high-affinity L-glutamate binding sites in *Caenorhabditis elegans*, but we were not able to detect any specific binding to *H. contortus* membranes. We were able to repeat the observations of Schaeffer *et al.* (1990) on *C. elegans* membrane preparations (data not shown) with similar results.

Kinetic analysis of the association of L-glutamate to *H. contortus* membranes was consistent with a

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Table 1. Kinetics of [^{3}H]L-glutamate binding to *Haemonchus contortus* membranes: dissociation constants for a 2 site model

	K_{fast}		K_{slow}	
	Rate constant	$t_{1/2}$	Rate constant ($\times 10^{-3}$)	$t_{1/2}$
Sensitive	$0{\cdot}84 \pm 0{\cdot}01$ min^{-1}	$0{\cdot}825 \pm 0{\cdot}02$ min	$3{\cdot}7 \pm 0{\cdot}2$ min^{-1}	$189{\cdot}3 \pm 25{\cdot}2$ min
Resistant	$0{\cdot}85 \pm 0{\cdot}02$ min^{-1}	$0{\cdot}831 \pm 0{\cdot}03$ min	$3{\cdot}7 \pm 0{\cdot}3$ min^{-1}	$191{\cdot}6 \pm 30{\cdot}5$ min

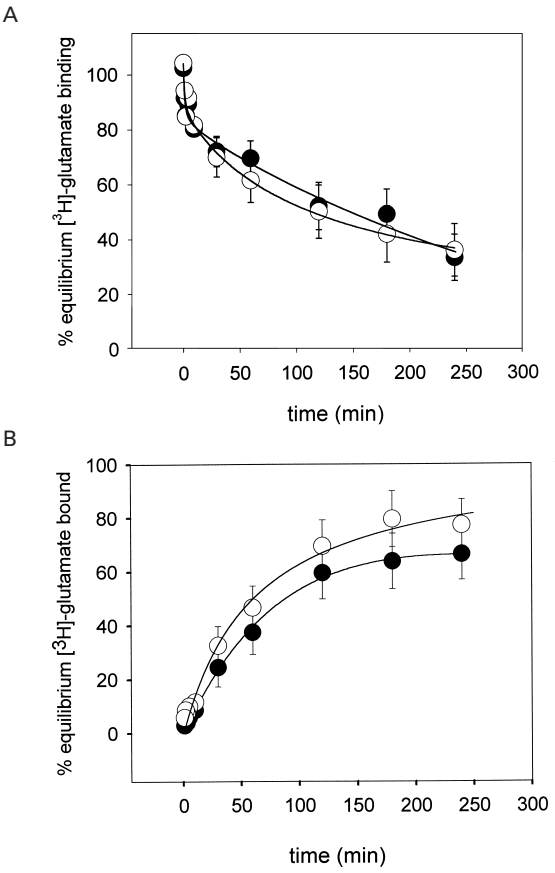

Fig. 3. Kinetics of L-glutamate binding to P2 membranes of ivermectin-sensitive (○) and resistant (White River) (●) *Haemonchus contortus*. (A) Dissociation kinetics. (B) Association kinetics.

single site with an association rate (K_{on}) of 78·23 min/μM, corresponding to a half-time ($t_{1/2}$) of 76 min (Fig. 3). The dissociation kinetics suggested a 2-site model with an extremely rapid minor population displaying a rate constant for dissociation of 0·84 per min corresponding to a $t_{1/2}$ of only 0·825 min, together with a second, larger, population with a rate constant of $3{\cdot}7 \times 10^{-3}$ per min and a $t_{1/2}$ of 189·3 min (Table 1).

Glutamate binding to the White River isolate of ivermectin-resistant H. contortus

When we repeated the [^{3}H]L-glutamate binding experiments using P2 membrane preparations from the White River isolate of ivermectin-resistant *H. contortus*, we observed a large increase in the number of the low-affinity specific binding sites (Fig. 2). In these experiments the observed B_{max} was $519{\cdot}1 \pm 68{\cdot}9$ pmol/mg of protein (3·5-fold higher than in the susceptible isolate) and the calculated K_d was $8{\cdot}74 \pm 2{\cdot}4$ μM (3·3-fold higher than in the susceptible isolate, but again the error is large). The Hill coefficient was again close to unity ($n_H = 0{\cdot}98$) and the results of the kinetic analysis were very similar to those obtained with the susceptible isolate. A comparison of the data obtained from the 2 isolates suggested that the major difference between them was a dramatic increase in the number of the low-affinity specific L-glutamate binding sites observed in the ivermectin-resistant worms.

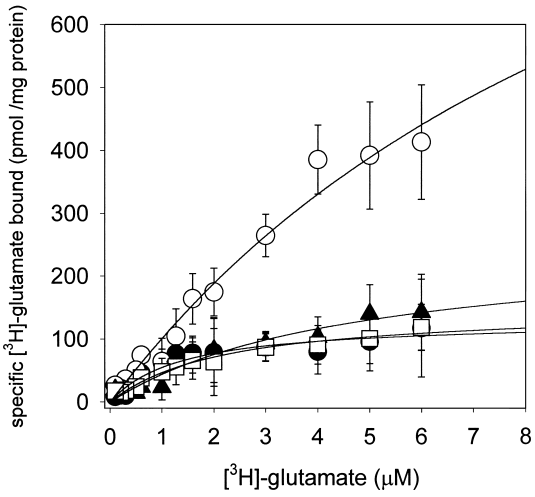

Fig. 4. Equilibrium binding of [^{3}H]L-glutamate to P2 membranes of ivermectin-sensitive (●) and resistant *Haemonchus contortus* Langford passage 2 (□); Langford passage 3 (▲); Langford passage 4 (○).

Specific L-*glutamate binding sites in experimentally produced ivermectin-resistant* H. contortus

The difference in the number of glutamate binding sites observed between the 2 isolates of *H. contortus* could have been due to natural variation between the isolates rather than the ivermectin-resistance phenotype. The genome of *H. contortus* shows a very high level of polymorphism and heterogeneity between individual worms (Hoekstra *et al*. 1997) and the 2

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Table 2. [^{3}H]L-glutamate binding to nematode membranes

	K_d (μM)	R/S (K_d)	B_{max} (pmol/mg protein)	R/S (B_{max})
Haemonchus contortus IVM-S	2·64 ± 1·0		148·3 ± 21·3	
H. contortus IVM-R White River	8·74 ± 2·4	3·31	519·1 ± 68·9	3·50
H. contortus IVM-R Langford4	12·1 ± 1·2	4·58	1331·5 ± 356·2	8·98
Telodorsagia circumcincta IVM-S	2·52 ± 0·81		17·08 ± 2·89	
T. circumcincta IVM-R	4·64 ± 1·12	1·84	31·52 ± 5·81	1·85

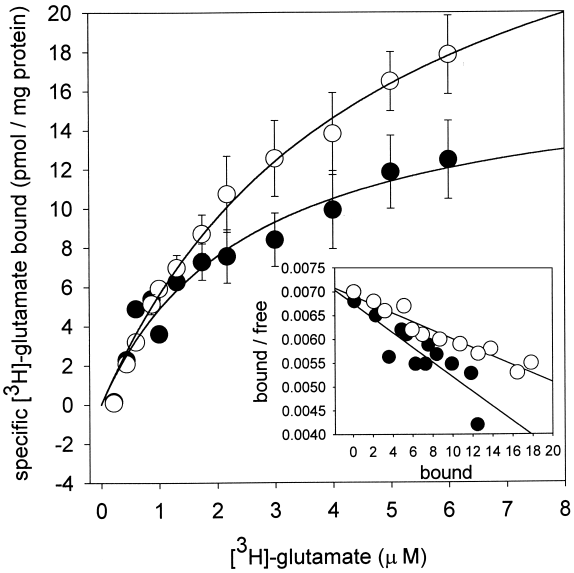

Fig. 5. Equilibrium [^{3}H]L-glutamate binding to P2 membranes from ivermectin-sensitive (●) and resistant (○) *Telodorsagia circumcincta*.

isolates used in the experiments described above were from geographically distinct regions (UK and South Africa). The White River isolate is also resistant to benzimidazoles, not just ivermectin (van Wyk & Malan, 1988). We therefore measured L-glutamate binding sites in 3 experimentally produced isolates, Langford 2–4, derived from our susceptible isolate. The Langford 4 isolate was fully resistant to a normal therapeutic dose (0·2 mg/kg); Langford 2 and 3 were resistant to subtherapeutic doses of the drug. The regime used to produce these isolates and their detailed characterization will be described elsewhere.

Specific [^{3}H]L-glutamate binding was measured to P2 membrane preparations of each of these isolates (Fig. 4 and Table 2). There was no significant difference in [^{3}H]L-glutamate binding to membranes from the susceptible, Langford 2 or Langford 3 worms, but membranes from the Langford 4 isolate showed an increase in B_{max} to 1331·5 ± 356·2 pmol/mg protein compared to 127·9 ± 28·6 pmol/mg protein in the original isolate – an increase of 10·4-fold. The apparent K_d increased from 1·3 ± 0·4 μM to 12·1 ± 1·2 μM – a 9·3-fold increase. This result was similar, in fact even more dramatic, to that obtained with the White River II isolate and suggested that the increase in the number of L-glutamate binding sites was very likely to be associated with the acquisition of ivermectin resistance in this species.

L-Glutamate binding to P2 membranes from Telodorsagia circumcincta

In order to examine whether the increase in low-affinity L-glutamate binding sites associated with ivermectin resistance was confined to *H. contortus* or was a more general phenomenon, we studied [^{3}H]L-glutamate binding to P2 membrane preparations from susceptible and resistant L3 larvae of *T. circumcincta* (Fig. 5). A similar low-affinity specific L-glutamate binding site was observed. In this species, the B_{max} for binding was markedly lower than in *H. contortus*, at 17·08 ± 2·89 pmol/mg protein, though the K_d was similar (2·52 ± 0·81 μM). In the resistant isolate both the B_{max} and the K_d increased by approximately 85% (B_{max} = 31·52 ± 5·81 pmol/mg protein; K_d = 4·64 ± 1·12 μM). Though the increase in the binding sites was not as dramatic as observed in *H. contortus*, the results are consistent with the hypothesis that the two phenotypes are linked.

Pharmacology of the L-glutamate binding site

Competition binding assays were used to compare the pharmacology of the L-glutamate binding sites in ivermectin-sensitive and resistant (White River) *H. contortus* (Fig. 6). No significant differences were observed in the displacement curves for L-glutamate, kynurenic acid (a NMDA antagonist) or β-hydroxy-aspartate (a glutamate transporter inhibitor). The displacement of L-glutamate binding by ibotenic acid was different between the 2 membrane preparations (Fig. 6B). In the preparation from ivermectin-sensitive nematodes, the curve had 2

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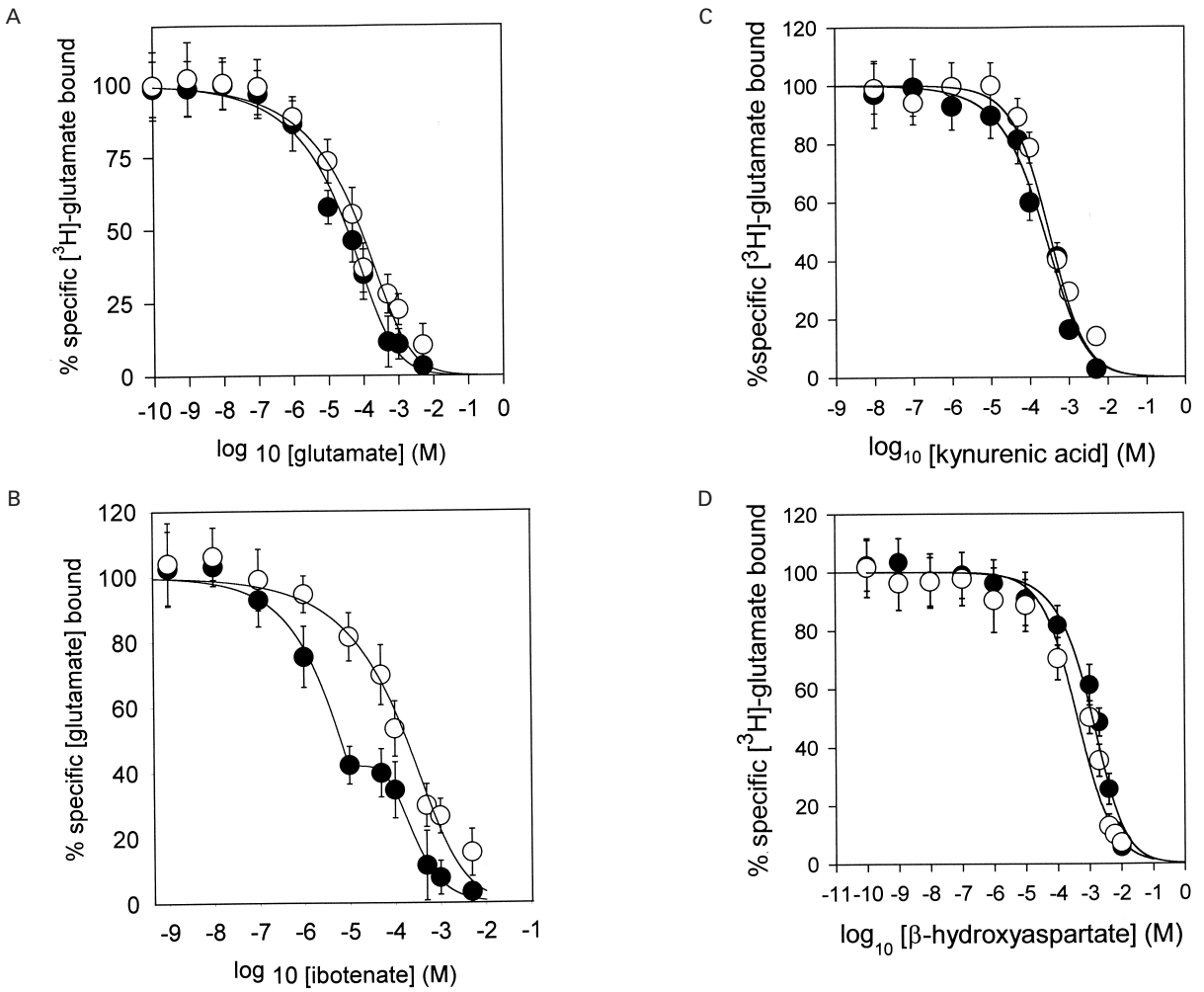

Fig. 6. Displacement of [^{3}H]L-glutamate binding from P2 membranes of ivermectin-sensitive (●) and resistant (White River) (○) *Haemonchus contortus*. (A) L-Glutamate. (B) Ibotenic acid. (C) Kynurenic acid. (D) DL-Threo β-hydroxyaspartate.

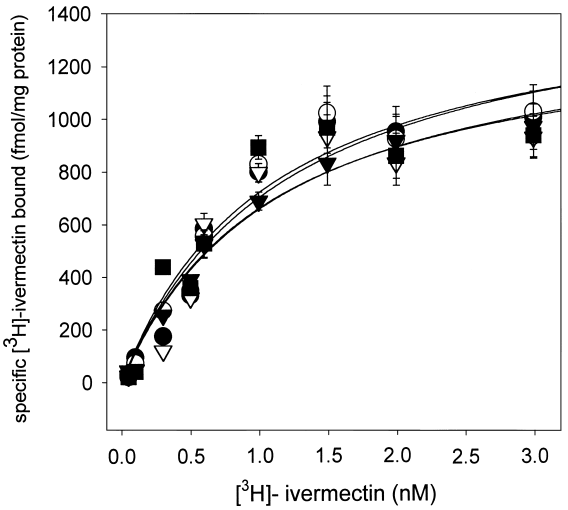

Fig. 7. [^{3}H]Ivermectin binding to P2 membranes of *Haemonchus contortus*. Ivermectin-sensitive (●), White River (■) and Langford (Passage 2, ▽; Passage 3, ▼; Passage 4, ○) isolates.

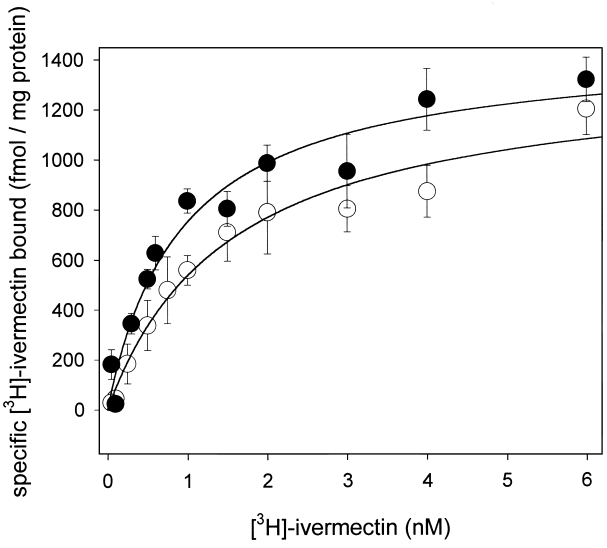

Fig. 8. [^{3}H]Ivermectin binding to P2 membranes of *Telodorsagia circumcincta* from ivermectin-sensitive (●) and resistant (○) isolates.

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Table 3. [^{3}H]Ivermectin binding to nematode membranes

	Haemonchus contortus IVM-S	*H. contortus* IVM-R White River	*H. contortus* IVM-R Langford4	*Telodorsagia circumcincta* IVM-S	*T. circumcincta* IVM-R
K_d (nM)	0·6 ± 0·1	0·7 ± 0·3	1·1 ± 0·2	1·5 ± 0·3	0·91 ± 0·2
B_{max} (pmol/mg protein)	1·38 ± 0·5	1·26 ± 0·5	1·49 ± 0·6	1·35 ± 0·4	1·44 ± 0·6

components with 60% of specific L-glutamate binding being displaced by ibotenate with an IC_{50} of approximately $1{\cdot}1 \times 10^{-6}$ M and the remaining 40% displaced with an IC_{50} of $1{\cdot}5 \times 10^{-5}$ M. When this experiment was repeated on membrane preparations from the White River isolate of ivermectin-resistant *H. contortus*, we could detect only a single population of L-glutamate binding site which was displaced by ibotenate with an IC_{50} of $9{\cdot}8 \times 10^{-4}$ M. Ivermectin, argiopine, kainate, quisqualate and NMDA all failed to inhibit L-glutamate binding to membrane preparations from either isolate.

Ivermectin binding to nematode membranes

Ivermectin is believed to act at glutamate-gated chloride channels (Arena *et al*. 1992; Cully *et al*. 1994). In order to determine whether the changes in specific L-glutamate binding we observed in ivermectin-resistant nematodes was due to changes in the ivermectin target site, we measured the binding of [^{3}H]ivermectin to P2 membrane preparations from all of the isolates used in the L-glutamate binding experiments (Figs 7 and 8). Equilibrium binding experiments revealed a high-affinity binding site in both *H. contortus* and *T. circumcincta* with a K_d of 0·6 ± 0·2 nM in *H. contortus* and 1·5 ± 0·3 nM in *T. circumcincta*. The observed B_{max} was 1·38 ± 0·5 pmol/mg protein in *H. contortus* and 1·35 ± 0·4 pmol/mg protein in *T. circumcincta*. No significant changes in any of these values were observed in any of the ivermectin-resistant isolates (Table 3).

Sequence and localization of putative glutamate-gated chloride channel subunits in the White River II isolate

Previously, we have reported the cloning and localization of 3 putative glutamate-gated chloride channel subunits from *H. contortus* (Delany *et al*. 1998; Jagannathan *et al*. 1999). We amplified and sequenced full-length cDNAs encoding all 3 subunits from the White River II isolate of ivermectin-resistant *H. contortus*. Three separate amplifications were carried out for each cDNA. When compared to the sequence of the cDNAs isolated from the susceptible isolate, no amino-acid coding changes were observed in any of the subunit cDNAs, though silent nucleotide variations were observed, consistent with the high level of third base polymorphisms observed in these genes (Delany *et al*. 1998). We also used anti-subunit antibodies to examine the expression of the 3 subunits in adult worms. In all cases the pattern of expression in the White River isolate was identical to that previously reported (Delany *et al*. 1998; Jagannathan *et al*. 1999).

DISCUSSION

These data confirm and extend the report of Paiement *et al*. (1999*b*) describing a high-abundance, low-affinity L-glutamate binding site on P2 membranes of parasitic nematodes. The observed K_d of 2·6 ± 1 μM correlates well with that (1·8 μM) of a membrane-associated glutamate binding protein from *Haemonchus contortus* observed by Rohrer, Evans & Bergstrom (1990). Although this site is clearly saturable, the low-affinity makes it difficult to measure the parameters of equilibrium binding with any accuracy and so all the K_d and B_{max} values quoted must be treated with some caution. Not only will binding require long incubations in order to reach equilibrium, but it is likely that some bound ligand will be lost during the washing procedure. Kinetic analysis of the association and disassociation rates show that, under the conditions used here, L-glutamate binding takes at least 2 h to come to equilibrium. The dissociation experiment reveals the presence of 2 binding sites, one of which has an extremely rapid off rate ($t_{1/2}$ = 0·825 min). This rapid off-rate makes it very likely that the majority of binding to this site will be lost during the washing procedure and may account for the difficulties obtained in reaching equilibrium conditions. Nevertheless, we believe it is possible to draw some conclusions about this site, despite the experimental difficulties involved. It is clear that the *H. contortus* larval membranes contain a very high number of these sites; the B_{max} is such that it is unlikely to represent any kind of neuroreceptor. The values for B_{max} and K_d we obtain are similar to those reported by Paiement *et al*. (1999*b*) for larval membranes.

When we compared the binding of L-glutamate to P2 membranes from ivermectin-resistant isolates of *H. contortus*, a clear increase in the number of the specific binding sites was apparent. The parameters of the equations that were fitted to the data suggested an increase in both the B_{max} and K_d of binding to the membranes from resistant worms, though it is

https://doi.org/10.1017/S0031182099005843 Published online by Cambridge University Press

possible that the apparent increase in K_d is an artefact caused by the failure of the binding to fully reach equilibrium. A similar difference was observed in both the White River II field isolate and the Langford 4 laboratory isolate of resistant worms. The Langford ivermectin-resistant isolates were derived from the susceptible isolate used in this study and had been passaged only 3–4 times in sheep (G. C. Coles, unpublished). These results exclude the possibility that the difference in L-glutamate binding sites between isolates is due to a natural genetic variation between the African and European isolates that is irrelevant to ivermectin resistance in *H. contortus*. Taken together with the similar observation of Paiement *et al.* (1999*b*), our results also suggest that an increase in L-glutamate binding sites will result regardless of the procedure used for inducing ivermectin resistance. The Merck and Fort Dodge isolates used by Paiement *et al.* (1999*b*) and others were generated using gradually increasing subtherapeutic doses of ivermectin, the Langford isolates were generated using rapidly increasing doses of ivermectin in only 3 passages and the field isolate was presumably generated using a single dose at therapeutic levels.

We studied the pharmacology of the L-glutamate binding site in both the susceptible and White River II isolates using competition binding assays. These showed that ibotenic acid and kynurenic acid along with β-hydroxyaspartate (a glutamate transporter inhibitor) were able to completely displace L-glutamate from these sites, though only at rather high concentrations. Ivermectin and other glutamatergic compounds had little or no effect on glutamate binding. The results for ibotenic acid were interesting. The inhibition curve suggests the possibility of 2 sites in the ivermectin-sensitive membranes, but only 1 in the ivermectin-resistant preparations. One explanation for this is that the increased total number of sites in the resistant preparation is solely due to the lower affinity site for ibotenate, and the higher affinity site represents too small a proportion of the total population to be readily detected in this assay. If the higher affinity site is approximately 60% of the glutamate binding site in membranes from ivermectin-sensitive nematodes, this would represent a B_{max} of about 89 pmol/mg protein: this level of binding would represent only about 17% of the total L-glutamate sites on the membranes from resistant nematodes and it is possible that this would not have been detected in the competition assay.

Blackhall *et al.* (1998) claimed to have detected selection at a glutamate-gated chloride channel gene (Forrester *et al.* 1999) in ivermectin-resistant *H. contortus*. If upregulation of such a channel was responsible for the increase in L-glutamate binding seen here, then we would predict that a similar increase may also be observed in ivermectin binding sites. However, we could detect no change in ivermectin binding sites in any of the *H. contortus* or *T. circumcincta* isolates tested, confirming the results of Rohrer *et al.* (1994). The number of ivermectin binding sites present on P2 membranes of *H. contortus* is 100-fold less than the number of glutamate binding sites making it very unlikely that the two are related. We also failed to detect any difference in the predicted amino-acid sequence or expression pattern of the HG2, HG3 and HG4 *H. contortus* L-glutamate-gated chloride channel subunits (Delany *et al.* 1998; Jagannathan *et al.* 1999). The absence of any changes in the amino-acid sequence on the subunits does not preclude the possibility that the pentameric Glu-Cl in the resistant worms has a different subunit composition from that in sensitive isolates. Since the *C. elegans* genome contains 5 genes encoding Glu-Cl subunits and only 3 have so far been identified in *H. contortus*, it is possible that mutations in genes yet to be identified may be involved in ivermectin resistance in parasitic nematodes. It is also possible that the sequence of the subunits expressed in eggs is not the same as that expressed in adult worms, as some of these genes are alternatively spliced. However, it is difficult to see how such mutations would lead to the large increase in glutamate-binding sites we have observed. We would therefore conclude that the increase in L-glutamate binding sites seen in ivermectin-resistant *H. contortus* is not related to any change in the L-glutamate-gated chloride channels that are the drug target site.

Other possible L-glutamate binding sites include excitatory receptors and amino-acid transporters. Davis (1998*a*) has demonstrated the existence of excitatory kainate-like receptors in *Ascaris suum* and such receptors have been studied in *C. elegans* (Hart, Sims & Kaplan, 1995; Maricq *et al.* 1995). Kainate and quisqualate were both agonists at the receptors described by Davis (1998*a*), so their failure to block L-glutamate binding in our experiments indicates that the binding site described does not represent the nematode kainate receptor. Glutamatergic synaptic transmission is dependent on inactivation of the transmitter via its removal by re-uptake transporters (Nicholls & Attwell, 1990). Davis (1998*b*) has provided pharmacological evidence for a glutamate transporter in *A. suum*: L-glutamate and L-aspartate were both agonists at concentrations above 100 μM, but NMDA, AMPA, quisqualate, domoic acid and kainate had little or no effect. DL-Threo-β-hydroxyaspartate and L-trans-pyrrolidine-2,4-dicarboxylate both activated the transporter when applied at 10 mM concentrations. These results are similar to the pharmacology of the L-glutamate binding sites we describe here, which are blocked by β-hydroxyaspartate, but not kainate, quisqualate or NMDA, raising the possibility that an upregulation of glutamate transporters might be involved in

https://doi.org/10.1017/S0031182099005843 Published online by Cambridge University Press

ivermectin resistance. If the action of ivermectin at therapeutic doses is to potentiate the normal activity of glutamate, then increased activity of transporters could lead to a reduction in the concentrations of the transmitter in synaptic clefts and elsewhere and thus a toxic effect of the drug is less likely. Such effects could account for the reduced sensitivity of pharyngeal pumping to ivermectin inhibition observed by Kotze (1998) and Paiement *et al*. (1999*a*).

It is important to know whether similar mechanisms of ivermectin resistance will arise in all parasitic nematodes or whether different strategies will be evolved by different species. Our experiments with *T. circumcincta* yielded somewhat equivocal results. Though a similar low-affinity L-glutamate binding site was detected in P2 membranes from L3 larvae of this species, it was present at much lower levels than in *H. contortus*. The increase in the number of the binding sites in the resistant isolate was also much less dramatic (only 85 % instead of > 450 %) though the number of ivermectin binding sites did not change. It will be interesting to compare L-glutamate binding sites from a range of ivermectin-resistant and susceptible nematodes in order to determine whether an increase in the low-affinity binding site is a common function and, if so, the size of that increase.

All of the binding experiments were carried out on parasite larvae. Selection for anthelminthic resistance presumably acts on the adult, so it is possible that any changes seen in larvae may not directly correlate with the mechanism of resistance in adults. However, most laboratory tests for resistance are carried out on the larval stages (Gill *et al*. 1991, 1995) and their reliability would suggest that our use of larval membranes is justified on scientific as well as economic and animal welfare grounds.

In conclusion, we believe that we have demonstrated a convincing correlation between an increased number of low-affinity L-glutamate binding sites on nematode membranes and ivermectin resistance in *H. contortus*. We are continuing to attempt to determine the molecular nature of these sites.

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). S.J. was supported by a University of Bath Research Scholarship and N.S.D. was supported by a BBSRC research studentship.

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