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HAEMONCHUS CONTORTUS: THE ROLE OF TWO β-TUBULIN GENE SUBFAMILIES IN THE RESISTANCE TO BENZIMIDAZOLE ANTHELMINTICS

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Abstract—The role of β-tubulin genes in benzimidazole (BZ) resistance was investigated using one susceptible (S) and two resistant (Rt and Rc) strains of *Haemonchus contortus*. The Rt strain was isolated from the field on the basis of thiabendazole resistance. The Rc strain was derived from the S strain by treatment with cambendazole. cDNAs, derived from the S strain, encoding two isoforms of β-tubulin (β12-16 and β8-9), α-tubulin and phosphofructokinase (Pfk) were used as probes for Southern hybridization analysis of genomic DNA digested by restriction enzymes. Genomic DNA was isolated from a pool of worms or single worms. The restriction-enzyme fragment length polymorphism (RFLP) differences among these strains depended on the enzyme and the probe used. When digested with *Stu* I or *Hpa* I, and probed under stringent conditions with β8-9 or β12-16, fewer fragments were seen in the Rt and Rc strains than in the S strain. Different hybridizing fragments were found in different individuals. The frequency of individuals bearing certain fragments hybridizing to β12-16 or β8-9 in the susceptible population was reduced significantly in the resistant populations. Some differences in RFLP between these strains were observed when probed with α-tubulin or Pfk, but the changes were not consistent with fragments being lost from the resistant strains as observed for β-tubulin probes. These changes in RFLP pattern correlate with changes in the binding profiles of BZs and isoelectric isoform patterns reported previously for these strains. The data confirm that reduced heterogeneity within the population is associated with BZ resistance. Our results show that both the β8-9 and the β12-16 subfamilies of β-tubulin are affected to a similar extent by this reduction in heterogeneity in a resistant population.

Key words: Haemonchus contortus; nematode; β-tubulin genes; benzimidazole; anthelmintic; drug resistance

Helminth infections in humans and other animals are controlled mainly by treatment with anthelmintics [1]. Anthelmintics have been used with great success in the past, but their frequent use in animals has resulted in resistance to BZs§ and other broad spectrum and some narrow spectrum anthelmintics [2–6]. Although anthelmintic resistance has been reported in many nematodes in sheep, goats and horses, BZ resistance in *Haemonchus contortus* in sheep has been of the greatest concern [5, 6]. The evidence available suggests that the mode of action of BZs, as anthelmintic, antifungal and antimitotic agents, involves binding to tubulin which disrupts the polymerization of tubulin to microtubules [7–10]. BZs selectively interact with tubulin with high-affinity binding [11, 12]. BZ resistance is associated with a loss of high-affinity receptors [11] and high-affinity binding correlated with the known anthelmintic potency of BZs [13], but the exact site of BZ binding is unknown. There are α- and β-tubulin isoforms in mammals and nematodes [14, 15], and it is not known which tubulin isoform(s) is associated with BZ resistance or whether both α- and β-tubulin take part in forming the binding site. Other studies in fungi (including yeast), *Caenorhabditis elegans* and *H. contortus* suggest that alteration in tubulin may account for BZ resistance [16–22]. Lubega and Prichard [23] compared the α- and β-tubulin isoform patterns of the susceptible (S) and resistant (Rt) strains of *H. contortus* by western blot analysis and observed that BZ resistance was associated with an alteration of the β-tubulin isoform pattern, whereas the α-tubulin isoform pattern of the S and Rt strains were similar. However, the S and Rt strains were of different genomic background. In this study, a resistant strain (Rc) of the same genomic background as the S strain was used for comparison.

Roos *et al.* [22] used a genomic probe specific to one β-tubulin isoform to analyze RFLP of *H. contortus* DNA and correlated BZ resistance in *H. contortus* with that isoform of β-tubulin. Two distinct cDNA clones of β-tubulin isoforms (β8-9 and β12-16) were isolated from the S strain [24]. The sequenced

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§ Abbreviations: BZ, benzimidazole; RFLP, restriction-enzyme fragment length polymorphism; ORF, open reading frame; MBZ, mebendazole; OBZ, oxibendazole; TBZ, thiabendazole; CBZ, cambendazole; S, benzimidazole-susceptible; Rt, TBZ-resistant; Rc, CBZ-resistant; Pfk, phosphofructokinase; EC_{50}, effective concentration of drug causing 50% egg-hatch inhibition; B_{max} = maximum drug bound at infinite drug concentration; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

β8-9 cDNA is nearly identical to the ORF of the genomic probe used by Roos *et al.*; they differ by a single nucleotide change leading to a single amino acid change. Geary *et al.* [24] digested genomic DNA of the S and Rt strains with *Eco*R I, *Spe* I, *Sph* I or *Hind* III and probed with β8-9 or β12-16. They were unable to correlate the differences in RFLP with BZ resistance as reported by Roos *et al.*, although the RFLP pattern of the BZ-resistant strain differed from that of the sensitive strain depending on the enzyme used [24]. The experiments reported in this paper were undertaken to reconcile the different results and to correlate changes in RFLP patterns with changes in the binding profiles of BZs.

MATERIALS AND METHODS

Parasites

One BZ-susceptible (S) and two BZ-resistant (Rt and Rc) strains of *H. contortus* were used. The S strain had no record of exposure to BZs, while the Rt strain was isolated from the field on the basis of TBZ resistance.* The S and Rt strains were characterized previously using egg-hatch and specific BZ-binding assays and tubulin analysis [11, 12, 23]. The Rc strain was derived from the S strain [25] by treatment with CBZ and was found to be cross-resistant to all BZs tested [26].

Egg-hatch and BZ-binding assays

The resistance status of the Rc strain was confirmed by comparison with the S and Rt strains using the egg-hatch and the specific BZ-binding assays. Egg-hatch assays using eggs from the susceptible and resistant populations were performed, and the drug concentration that reduces egg hatch by 50% (EC_{50}) was determined as described [27]. BZ-binding assays were performed, and B_{max} values (maximum drug bound at infinite ligand concentration) were determined as described [12].

cDNA probes

The probes were the cDNAs encoding β-tubulins (β12-16 and β8-9 isoforms) [24], Pfk [28], and α-tubulin [29] isolated from a library constructed from the S strain.

Preparation of DNA

Adult *H. contortus* were recovered from sheep as described previously [12]. Genomic DNA was isolated from 3–5 g of adult worms using the proteinase K method [30].

DNA was isolated from single male worms as described [22]. Female worms were not used to avoid contamination from the eggs.

Digestion of DNA with restriction enzymes

*Eco*R I, *Hpa* I or *Stu* I were used to digest the genomic DNA as recommended by the supplier (New England Biolabs Ltd., Missisauga, Ontario, Canada). A sample of undigested DNA from each strain was routinely included and handled in the same manner as the digested samples to ensure that the digestion pattern was specific for the enzymes used.

Southern hybridization

Southern hybridization was carried out using Hybond-N membrane following the protocol of the supplier (Amersham, Oakville, Ontario, Canada). About 20 μg of genomic DNA was fractionated through 0.7% agarose gel as described [30] and transferred to the Hybond-N membrane using a vacuum blotter (model 785, Bio-Rad) following the protocol of the manufacturer. The probes were labelled with [α-^{32}P] CTP (3000 Ci/mmol; ICN, St. Laurent, Quebec, Canada) using a random primer kit (Boehringer Mannheim, Laval, Quebec, Canada). Membranes were prehybridized for 1 hr at 65° in 6 × SSC containing 200 μg/mL denatured salmon sperm-DNA (Pharmacia). The ^{32}P-labelled probe was added and hybridized overnight at 65°. The membranes were washed at 65° for 2 × 15 min in 2 × SSC, for 2 × 15 min in 0.1% (w/v) SDS in 2 × SSC, and for 10 min in 0.1% (w/v) SDS in 0.1 × SSC. Autoradiography was carried out using Kodak diagnostic film (Rochester, NY, U.S.A.) in a cassette with an intensifying screen (Fisher, Montreal, Quebec, Canada) at −70° for 1–4 days. Each membrane was probed sequentially with the four probes following removal of the preceding probe.

Southern hybridization analysis of genomic DNA from single worms was carried out as described above, with 1 μg of pooled genomic DNA run in the same gel as the control. To improve the signal, autoradiography of single-worm blots was carried out using two chemiluminescence films (Hyperfilm, Amersham) and two intensifying screens for 7 days at −80°. The two exposed films were carefully overlapped and photographed.

RESULTS

Comparison of strains by egg-hatch and specific BZ-binding assays

The EC_{50} values for TBZ and the B_{max} values for

Table 1. EC_{50}* and B_{max}† values of the S, Rt or Rc strains

Strain	EC_{50} ([TBZ] μM)	B_{max} values (pmol/mg protein)	
		[^{3}H]OBZ	[^{3}H]MBZ
S	1.5 ± 0.6	121 ± 7	101 ± 9
Rt	4.9 ± 0.5	31 ± 4	51 ± 5
Rc	7.4 ± 0.7	19 ± 3	32 ± 4

Values are means ± SEM, N = 6.

* The effective concentration of TBZ that inhibited the hatching of the eggs by 50%.

† Maximum high-affinity (specific) binding (HAB) at infinite ligand concentration of OBZ or MBZ in supernatants prepared from eggs of the S, Rt or Rc strains. Low-affinity binding (LAB) and total binding (TB) were determined, and B_{max} values for HAB (i.e. receptor concentration) were calculated using LIGAND as described in Ref. 12. For determination of LAB and TB, supernatants were incubated with the [^{3}H]BZ in the presence (LAB) or absence (TB) of 10 μM BZ for 45 min at 37°.

* Rew RS, personal communications. Cited with permission.

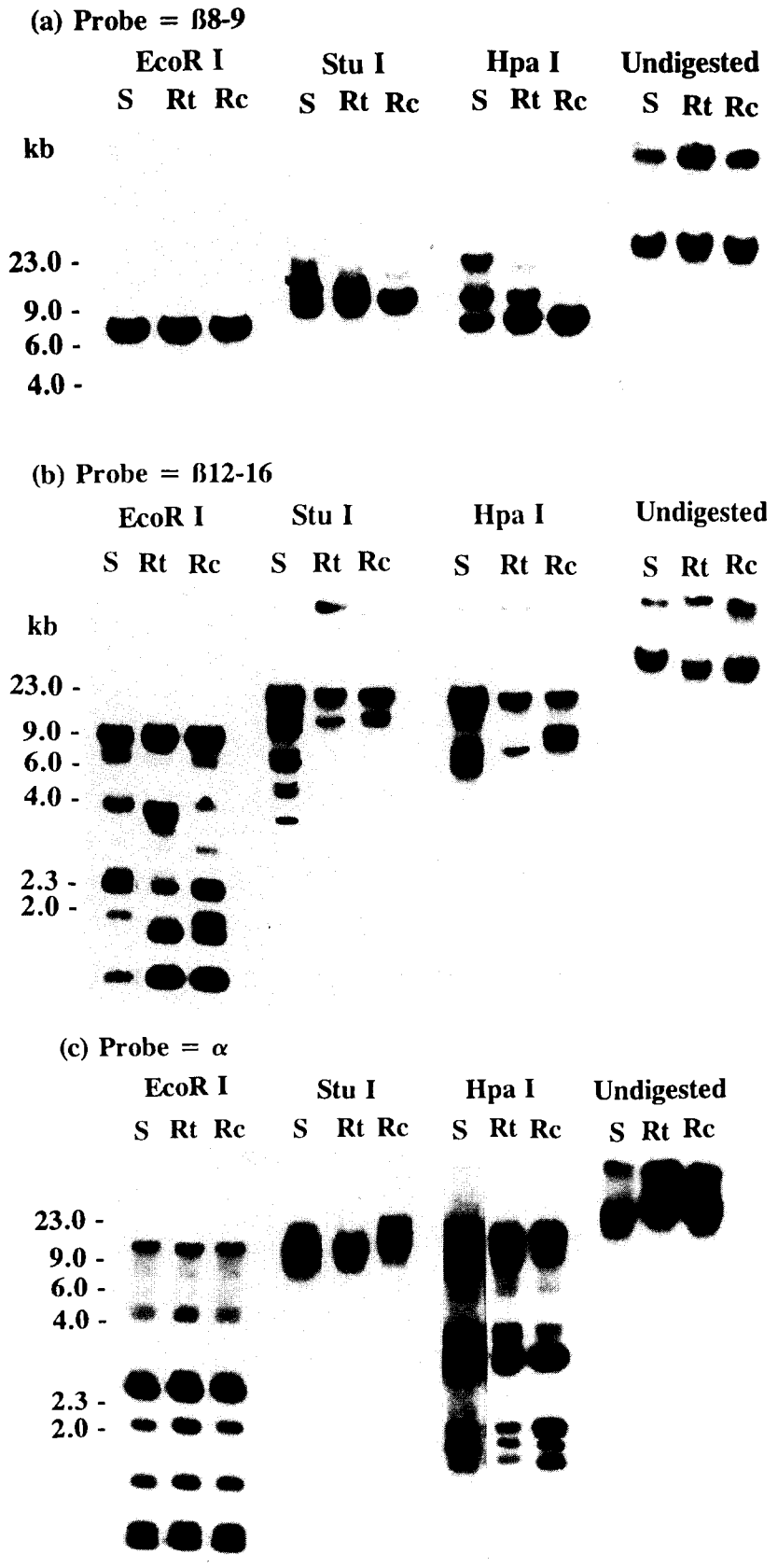

Fig. 1. (continued on next page).

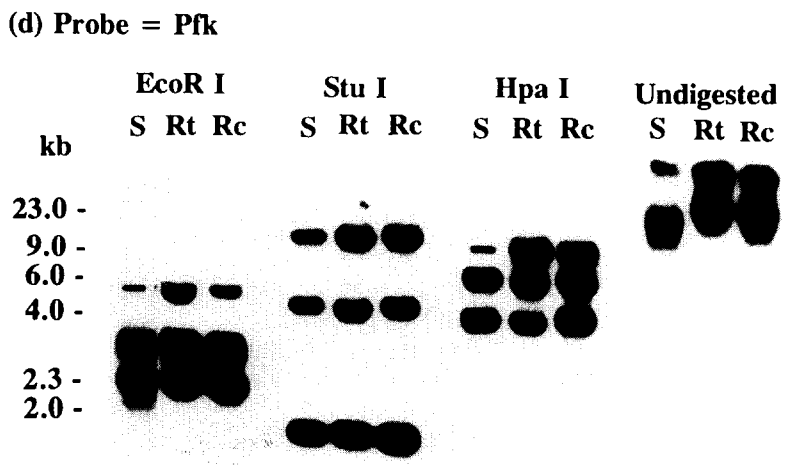

Fig. 1. RFLP analysis of genomic DNA isolated from a large number of worms of the S, Rt or Rc strains of *H. contortus*. Filters were hybridized with cDNAs encoding β8-9 (a), β12-16 (b), α-tubulin (c) or Pfk (d).

tritiated OBZ and MBZ are shown in Table 1. The binding of OBZ and MBZ was decreased in the resistant strains. By both the egg-hatch assay and extent of binding, the rank order of sensitivity was S ≥ Rt > Rc.

Southern hybridization using DNA from a large population of worms

β8-9 cDNA probe. The β8-9 and β12-16 cDNAs are 96.6% identical at the amino acid level but do not cross-hybridize at high stringencies in Southern blots due to a nucleotide change at most third positions of the codons as well as differences in amino acid sequence at the carboxy-terminus [24]. The banding pattern obtained after probing with β8-9 was simple and showed 1–3 major fragments (Fig. 1a). In *Eco*R I digests there was one major band at 7.5 kb in the three strains. Differences in hybridizing bands among the strains were seen in *Stu* I and *Hpa* I digests. The numbers of hybridizing fragments were fewer in the resistant strains than in the S strain. In *Stu* I digests, a doublet near 9 kb was seen in the S strain, but the upper band of the doublet was weaker in the Rt strain and absent in the Rc strain. Similar differences were seen in *Hpa* I digests. The S strain had three major bands around 7.5, 9.0 and 23 kb. In the resistant strains, the 7.5 kb band was predominant. The 23 kb band was weak in the Rt strain, whereas the 23 and 9 kb bands were missing from the Rc strain.

β12-16 cDNA probe. The banding pattern obtained after probing with β12-16 was generally more complicated than that with β8-9 (Fig. 1b). The clearest differences between the susceptible and resistant strains were demonstrated by the *Stu* I digests. Fewer hybridizing fragments were seen in the resistant strains than in the S strain. The S lane showed bands around 23.0, 9.0, 6.0, 5.5, and 3.5 kb. Not all of these bands were clearly visible in the Rt and Rc lanes. In the S lane, the bands at 9.0, 6.0 and 5.5 were doublets, whereas they were single or missing in the resistant lanes. The Rt and Rc strains were similar with two major bands (at 23.0 and 9.0 kb), although weak bands, similar to those seen in the S strain, appeared after prolonged exposure. Thus, the fragments were reduced in number and/or intensity in the resistant strains compared with the S strain. Differences between the sensitive and resistant strains were also seen in *Hpa* I digests. In *Hpa* I digests, the S strain had a band near 5 kb not seen in the Rt and Rc strains, and the Rc strain differed from the S and Rt strains by the presence of a band near 9.0 kb.

α-tubulin cDNA probe. The RFLP upon probing with α-tubulin cDNA is shown in Fig. 1c. The banding patterns for *Eco*R I and *Hpa* I in the S, Rt and Rc strains were identical except for differences in the intensity of some fragments. In *Stu* I digests, the S and Rt strains looked basically similar with the Rc strain being slightly different.

Phosphofructokinase (Pfk) cDNA probe. The banding pattern observed with the Pfk cDNA probe was unremarkable for the *Stu* I digests, but a difference between the S and the resistant strains was seen for *Eco*R I and *Hpa* I (Fig. 1d); the bands at 6.0 kb in the *Eco*R I digests and at 9.0 kb in *Hpa* I digests were faint in the S lane compared with the Rt and Rc lanes.

Southern hybridization using genomic DNA from individual worms

DNA from individual male worms was digested with *Stu* I or *Hpa* I, and the blots were probed with β12-16 or β8-9 cDNA (Figs. 2–4). Each strain had

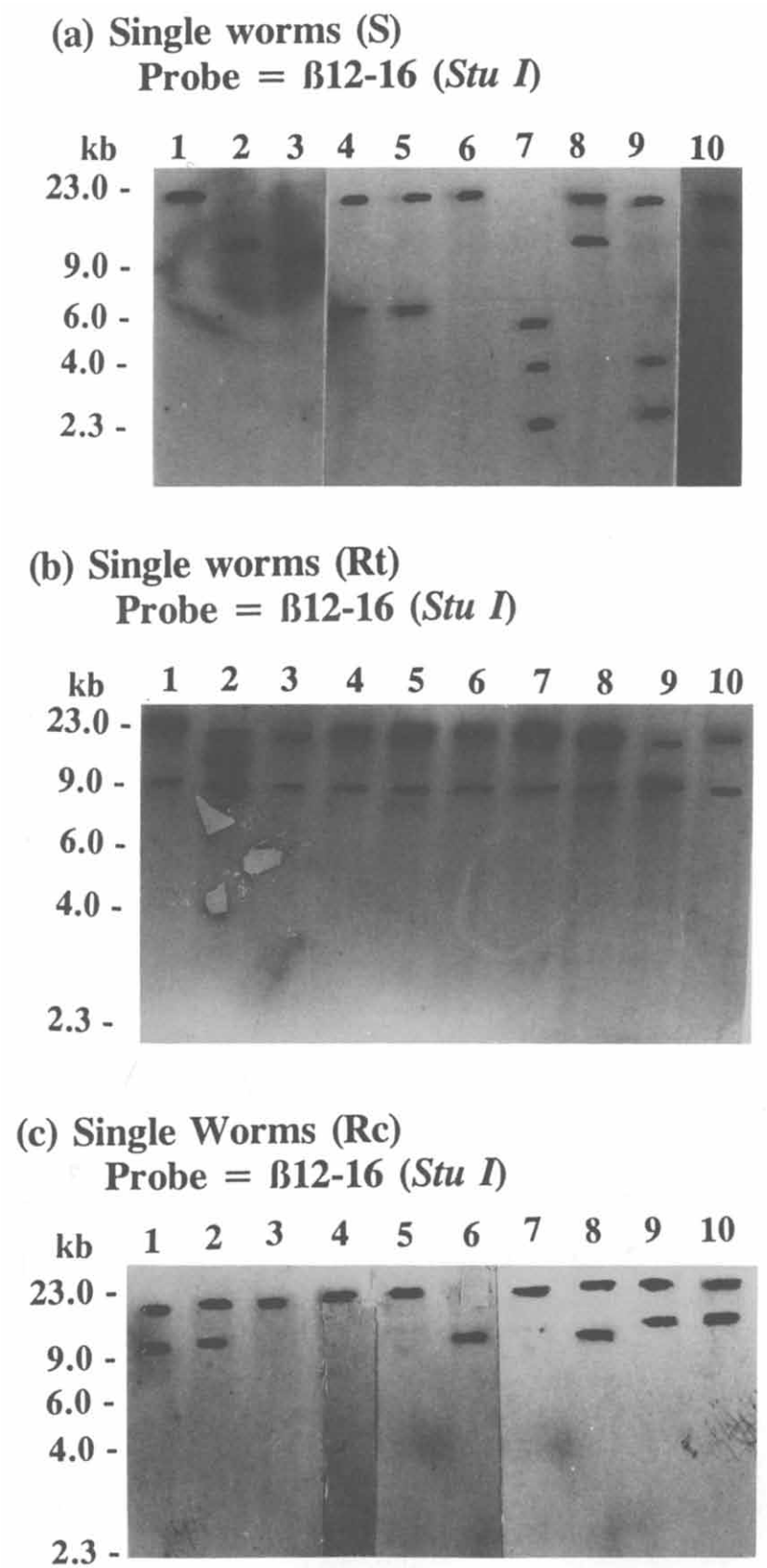

Fig. 2. RFLP analysis of β12-16 cDNA-hybridizing fragments in *Stu* I digested genomic DNA isolated from individual male worms of the S (a), Rt (b) and Rc (c) strains of *H. contortus*. Refer to Fig. 1b for comparison with blots of DNA isolated from a pool of many worms.

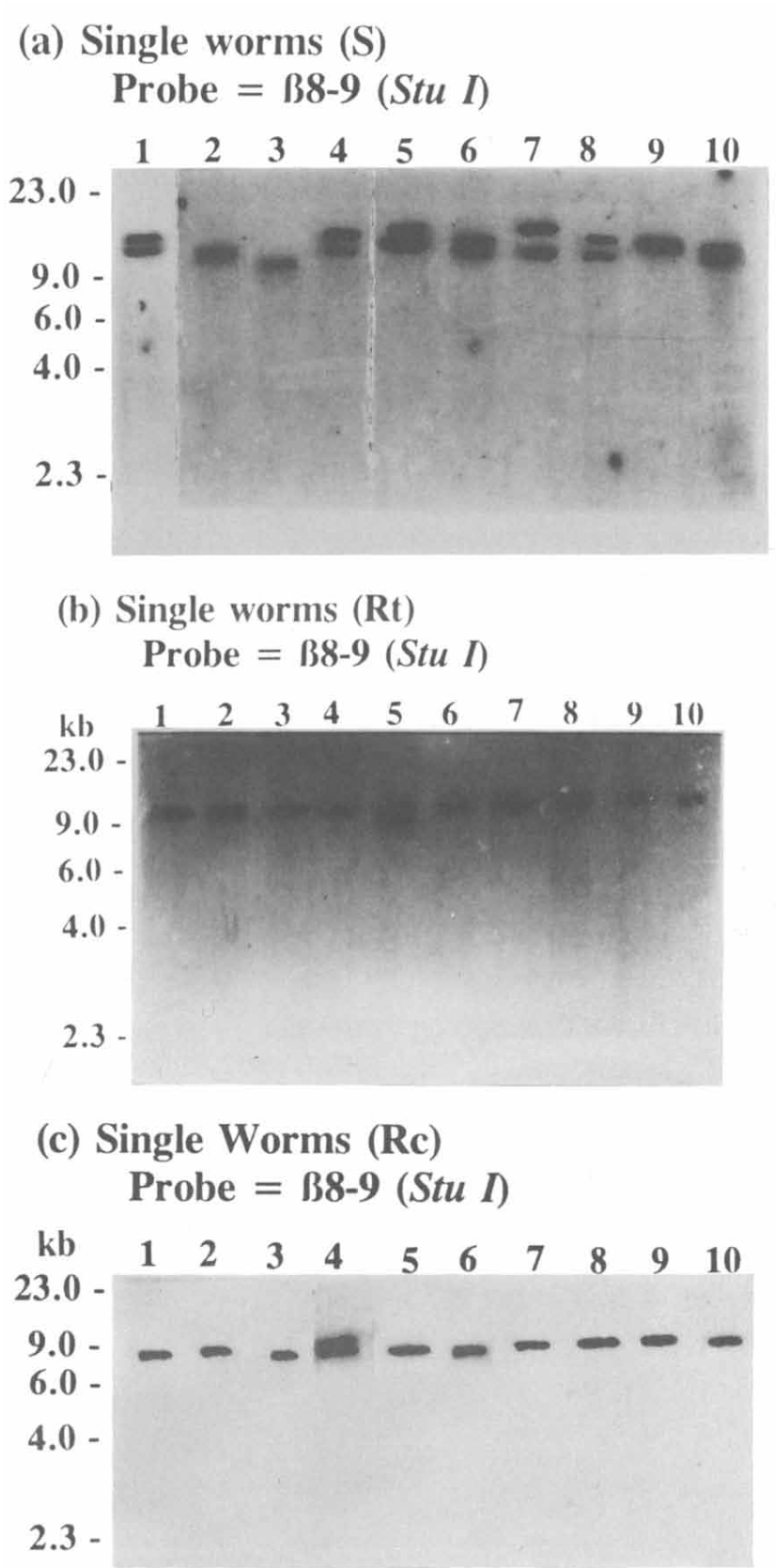

Fig. 3. RFLP analysis of β8-9 cDNA-hybridizing fragments in *Stu* I digested genomic DNA isolated from individual male worms of the S (a), Rt (b) and Rc (c) strains of *H. contortus*. Refer to Fig. 1a for comparison with blots of DNA isolated from a pool of many worms.

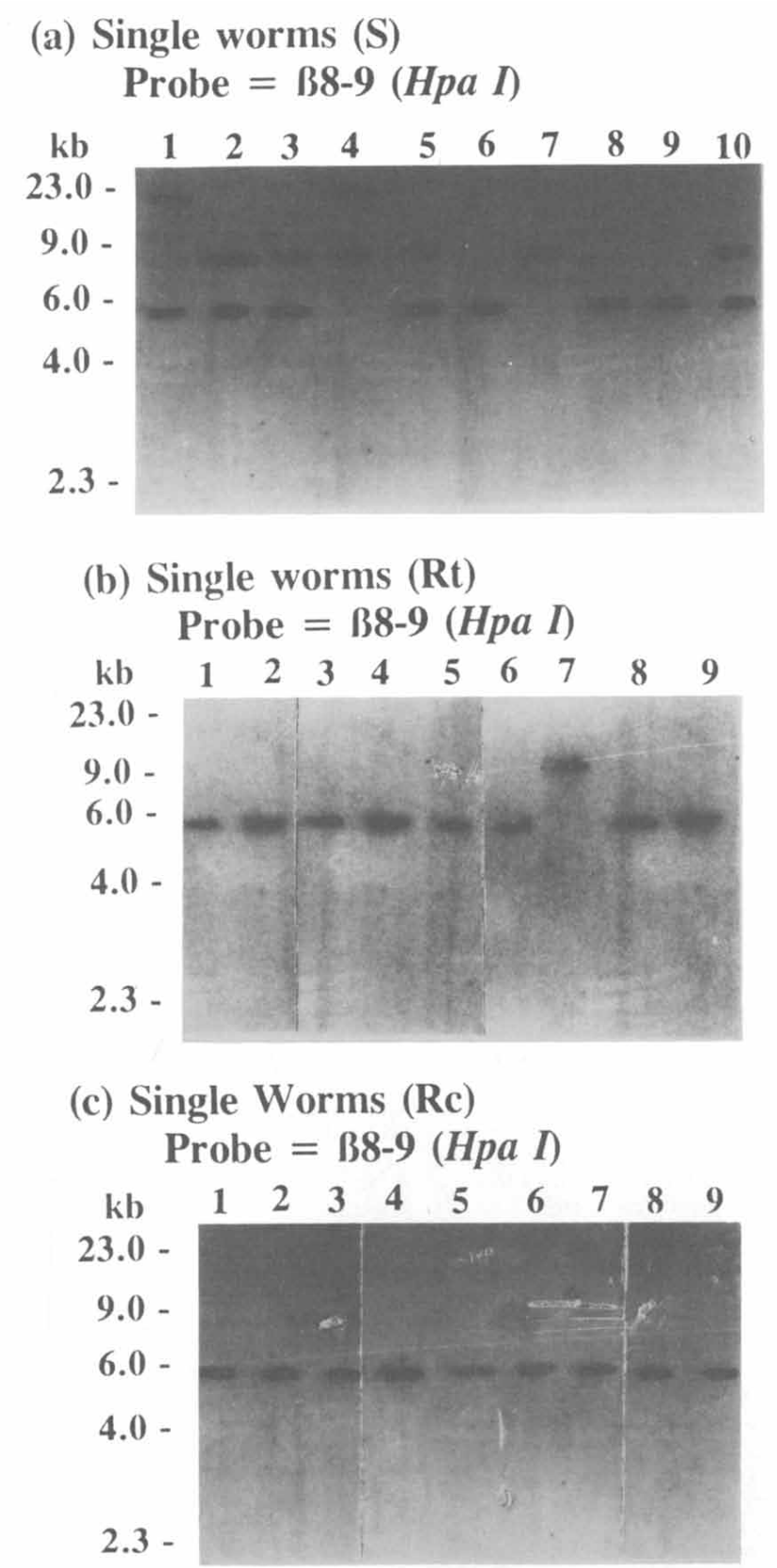

Fig. 4. RFLP analysis of β8-9 cDNA-hybridizing fragments in *Hpa* I digested genomic DNA isolated from individual male worms of the S (a), Rt (b) or Rc (c) strains of *H. contortus*. Refer to Fig. 1a for comparison with blots of DNA isolated from a pool of many worms.

a unique distribution frequency of the different RFLP patterns contained within the total population. The *Stu* I digests probed with β12-16 indicated that the S population was highly heterogeneous with respect to the hybridizing fragments (Fig. 2a). All eight fragments seen in the genomic DNA isolated from the total population of the S strain (refer to Fig. 1b or 5b) were represented in individual worms but in various combinations, indicating that the fragments can be segregated from each other. For example, worms in lanes 1 and 6 had similar single fragments, whereas lanes 2 and 3 contained different single fragments. Worms 7 and 9 contained 3 fragments but in different combinations. Whereas worms 4 and 5 were identical, they contained a combination of fragments different from those contained by worms 8 and 10, which were also identical.

Stu I digests probed with β12-16 indicated that the resistant populations (Fig. 2, b and c) were less heterogeneous than the S population. All the Rt worms, except the worm in lane 2, which had 3 bands, had the same 2 bands seen in the DNA from the total population of the Rt strain (Fig. 2b). Similarly, the Rc single worms (Fig. 2c) contained both or only one of the two predominant bands seen in the total population of the Rc strain.

Probing of the same *Stu* I digests with β8-9 showed that the S population (Fig. 3a) may be more heterogeneous than the resistant populations (Fig. 3, b and c). [Note that it was technically easier to resolve the fragments in individual worms than in pooled worms (refer to Fig. 1a).] Worms 1, 4, 5, 6, 7, 8 and 10 of the S strain contained 2 fragments, while worms 1 and 9 contained single fragments different from the single fragment of worm 3. On the other hand, the Rt worms (Fig. 3b) had identical single fragments except for worm 5, which contained 2 fragments. Similarly, the Rc worms (Fig. 3c) had identical single fragments except for worm 4, which had 2 fragments.

Hpa I digests probed with β8-9 indicated greater heterogeneity for the S strain (Fig. 4a) than for the resistant strains (Fig. 4b and c). The most intensely hybridizing bands in the pooled extracts (refer to Fig. 1) were the most frequently represented in individual worms. The less intensely hybridizing bands were represented less frequently. The three bands seen in the Southern blots of DNA from the total S population were represented in individual worms in various combinations of two bands (Fig. 4a). In the Rt population (Fig. 4b), 8 of the worms contained only a 6.0 kb band, while the other worm (lane 7) contained only a 9.0 kb fragment. All 9 Rc worms (Fig. 4c) contained only the 6.0 kb band.

Effect of treatment with CBZ or OBZ

The S and Rt strains were treated *in vivo* with CBZ (30 mg/kg body wt) or OBZ (10 mg/kg body wt) to see if the RFLP of the survivors would shift toward that of the Rc strain. The treatment almost completely eliminated the S strain but not the Rt strain (Table 2). Genomic DNA isolated from the Rt worms that survived CBZ (Tc) or OBZ (To) treatment was digested with *Stu* I or *Hpa* I and analyzed by Southern hybridization (Fig. 5). When digested with *Hpa* I and probed with β8-9 (Fig. 5a), minor changes were seen between the Rt and Tc or To lanes. When the same blot was probed with β12-16 (Fig. 5b), Pfk or α-tubulin (not shown) cDNAs, no significant changes were seen between the Rt and Tc and To lanes for either enzyme.

Table 2. Fecal egg counts per gram before and after treatment of the S or Rt strain with CBZ or OBZ in sheep

Strain	Treatment (mg/kg body wt)	Eggs per gram			
		Day 0	Day 3	Day 6	Day 10
S	CBZ (30)	21,211		5	11
	OBZ (10)	24,315			2
Rt	CBZ (30)	19,875	2750	5100	10,850
	OBZ (10)	15,650	1546	2550	13,500

DISCUSSION

It is believed that BZs exert their anthelmintic effects through binding to tubulin [2, 10]. The egg-hatch and the specific BZ-binding assays distinguished the S and Rt strains [11, 12]. The Rt strain was not of the same genetic background as the S strain. The Rc strain reported here was of the same genetic background as the S strain. Both the egg-hatch and BZ-binding assays showed that both Rt and Rc strains were resistant relative to the S strain. Furthermore, these assays suggested that the Rc strain was more resistant than the Rt strain.

When the S and Rt strains were compared, BZ resistance was associated with a loss of high-affinity BZ receptors from the eggs, larvae and adult worms [12]. This was associated with an alteration of β-tubulin, but not α-tubulin isoform patterns on two-dimensional PAGE [23]. Since the Rt strain was not derived from the S strain, the differences between them could have been caused by selection pressure due to the anthelmintic or by other factors. The Rc strain was derived from the S strain by treatment with CBZ [25].

In this study, we compared RFLP patterns of the S, Rt and Rc strains. After digestion with *Stu* I or *Hpa* I and probing with β8-9 or β12-16, fewer fragments were observed in DNA from pooled worms of the Rt and Rc strains than in that from the S strain. Prolonged exposure indicated that fragments had not disappeared but had become less intense. RFLP analysis of single worms showed that the apparent loss of fragments from the resistant populations was due to a reduced frequency of individuals bearing those fragments. There are no *Stu* I or *Hpa* I restriction sites in the cDNA of β8-9 or β12-16 [24]. Therefore, the various fragments may have been produced by cutting in the ORF of mutants of these genes or in introns or in flanking sequences of the same entire β8-9 or β12-16 genes. Analysis of single worms indicated that these fragments can be segregated into many combinations, suggesting that they may represent different alleles.

Differences in the number of β12-16 hybridizing

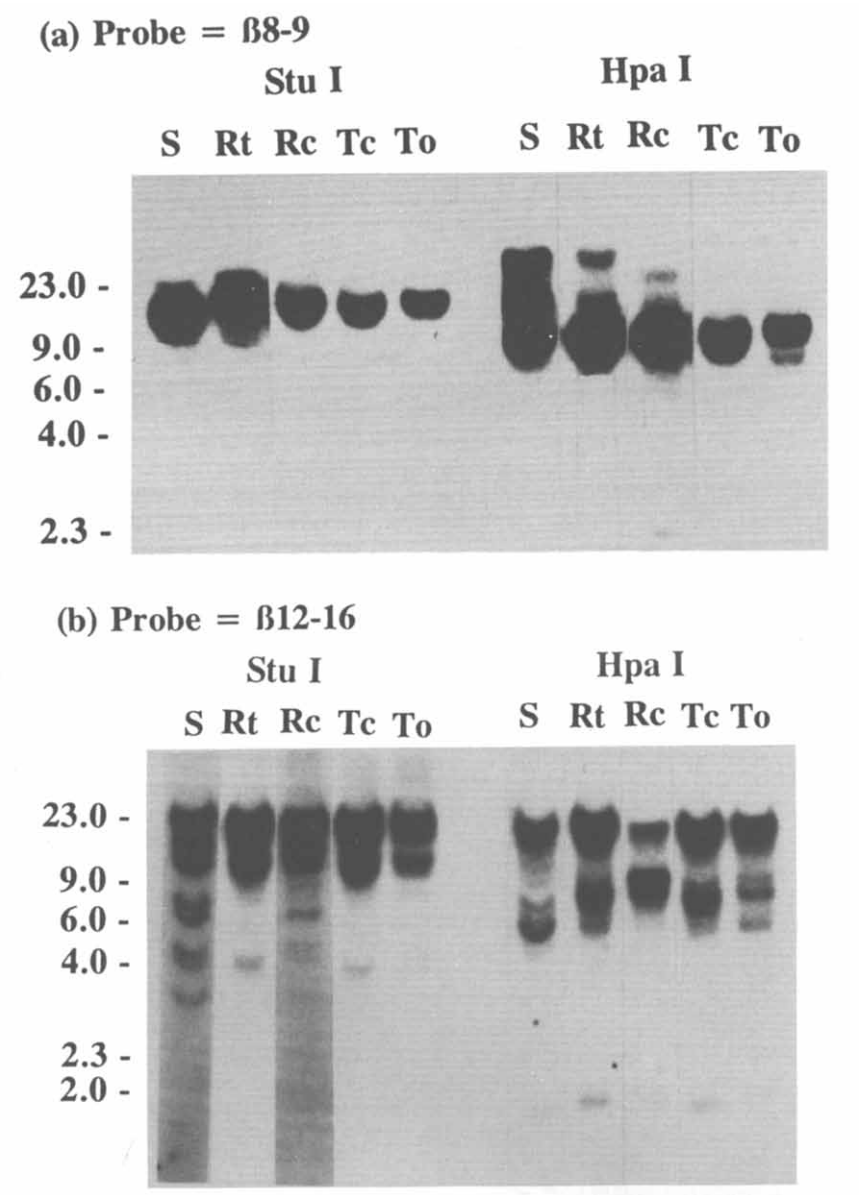

Fig. 5. RFLP analysis of the genomic DNA isolated from a large population of the survivors after treatment of the Rt strain with CBZ (Tc) or OBZ (To). The DNA was digested with *Stu* I or *Hpa* I and probed with cDNAs encoding β8-9 (a) or β12-16 (b). Genomic DNA from S, Rt and Rc strains was included for reference.

fragments between *Stu* I digests of the susceptible and the resistant strains were more dramatic than those observed with the β8-9 probe. Compared with the β8-9 probe, the banding pattern obtained with S worms probed with β12-16 was much more complicated. There is no indication of cross-hybridization between β8-9 and β12-16 [24]. The greater number of fragments seen with the β12-16 probe compared with the β8-9 probe suggests that there may be more β-tubulin alleles of β12-16 than of β8-9. A β-tubulin cDNA termed β12-164 that closely resembles β12-16 has already been isolated from the susceptible strain [24]. However, efforts to find additional β-tubulin clones have been unsuccessful.

A loss of individual worms bearing specific β-tubulin cDNA-hybridizing fragments is interesting given the results of pharmacological studies which show that BZ resistance is associated with a loss of high-affinity BZ receptors [11, 12]. The β-tubulin DNA fragments that are reduced in intensity in the resistant compared with the susceptible strains may be correlated with high-affinity binding. Our data shows that RFLPs for α-tubulin cDNA-hybridizing fragments were not correlated with BZ resistance. Roos *et al.* [22] observed no correlation between BZ resistance and the number of α-tubulin genomic-DNA hybridizing fragments. Other studies suggest that BZ resistance is associated with alteration of β-tubulin genes [reviewed in Refs. 2 and 24].

Our β8-9 data are consistent with those of Roos *et al.* [22], who used a genomic probe whose ORF was almost identical to our β8-9 cDNA. However, in our case only the enzymes *Stu* I or *Hpa* I, which apparently fail to cut the cDNA of β8-9 and β12-16, revealed differences between the susceptible and resistant strains. We also report that changes associated with β12-16 are similar to and seem to occur concurrently with those associated with β8-9. Recently, Kwa *et al.* [31] reported that at lower degrees of BZ resistance, selection of isotype 1 β-tubulin (similar to β8-9) takes place; subsequently, at higher degrees of drug resistance, an abrupt elimination of individuals carrying isotype 2 (similar to β12-16) takes place. The data reported here indicated that selection of both isotypes 1 and 2 takes place the same way and that this is not a dual phase mechanism. Studies on other susceptible and resistant populations are needed to determine the specific loci and alleles that are associated with selection for BZ resistance. BZ resistance in trichostrongylid nematodes behaves like a polygenic trait [32–34]. Multiple genetic changes involving β-tubulin genes may lead to BZ resistance.

Overall, our data suggest that reduced heterogeneity of both the β8-9 and β12-16 isotypes, within the population, is associated with selection of resistant strains. This selection hypothesis has been raised previously [4, 10, 11, 16, 22, 35]. The egg-hatch and the BZ-binding assay data suggest that the Rt strain was less resistant than the Rc strain. Similarly, RFLP analysis suggested that the variations in RFLP were greater in the Rt strain than in the Rc strain. When the survivors of CBZ and OBZ treatment were compared with the untreated Rt strain, minor alterations in the β8-9 RFLP were seen (see Fig. 5a). This would be consistent with the elimination of susceptible individuals in the population and would be supported by the observation that EPG (eggs per gram) counts remained depressed 10 days after treatment of the Rt strain with CBZ or OBZ (see Table 2).

Restriction enzymes, such as *Eco*R I, *Sph* I and *Hind* III, that cut the cDNA of β8-9 and β12-16 fail to produce a pattern that correlates with BZ resistance [24]. Neither *Stu* I nor *Hpa* I cut the β8-9 or β12-16 cDNA probes used. Further work is required to determine how the β8-9 and β12-16 families are involved with the receptors for BZs and which alleles of each of these subfamilies are lost in resistant individuals.

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