ARRESTED DEVELOPMENT OF *TRICHOSTRONGYLUS COLUBRIFORMIS* IN EXPERIMENTALLY INFECTED RABBITS. THE EFFECT OF ACQUIRED RESISTANCE, SINGLE, MULTIPLE DOSES AND SIZE OF DOSES ON RISE OF HYPOBIOSIS*

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Inhibited development of *Trichostrongylus colubriformis* was studied in single or multiple infected rabbits. Groups of 5 rabbits were infected with a single dose of 10 000 infective larvae of *Trichostrongylus colubriformis* per rabbit or lower multiple infections with or without immunosuppression. All rabbits were killed 20 days after last infection. Experiments are described which show that in a recently isolated field strain of the nematode, the major stimulus to the induction of inhibited development at the early third larval stage (EL_3) in the host in this study is repeated infection with 750 L_3 in 5-day-interval with immunosuppression. This infection resulted in a mean of 25.26% of the established worm burden becoming inhibited at the EL_3 stage. Other infection did not increase the proportion of worms subsequently found to be hypobiotic.

arrested development; *Trichostrongylus colubriformis*; multiple infection; immunosuppression

INTRODUCTION

An important consideration in parasite control through epidemiology is the hypobiotic or arrested development phenomenon, through which larvae survive in the host. Hypobiosis represents one of the most useful life cycle adaptations to ensure parasite survival and appears to be widespread among Trichostrongyloidea in ruminants and Cyathostominae in horses.

In previous work it has been shown that inhibition of *Trichostrongylus colubriformis* at the early third larval stage is a particular characteristic of the worms exposed to autumnal conditions.

Initially, hypobiosis was thought to be associated with acquisition of immunity (Martin et al., 1957; Michel, 1963; Soulsby, 1966). The larval inhibition associated with repeated infections have been observed by Donald et al. (1964) with *Nematodirus spathiger*, by Dineen and Wagland (1966) with *Haemonchus contortus*, by Roberts et al. (1962) with *Oesophagostomum radiatum* in sheep, by Michel (1963) and by Michel et al. (1973) with *Ostertagia ostertagi* and Roberts (1957) with *Haemonchus placei* in calves.

In the present project, the effects of size and frequency of infections with goat trichostrongylid *T. colubriformis* were examined in the experiments in the rabbit host-model.

MATERIAL AND METHODS

Model host

The rabbits (New-Zealand white race) of similar age (3 months) and weight, both sexes were kept in cages with wire-netting floors, and were fed on pellet-form diet KKK POLT with anticoccidium for the duration of study. Faecal examinations were made before infection to ensure that animals were free from natural infections.

Acquisition of parasites

Two 1/2 Bursiań x 1/2 Czech short hair white cross-bred male goat kids were treated per os with 10 $mg.kg^{-1}$ fenbendazole (Panacur, American Hoechst Corp.) at 2 months of age. Faecal examinations at the time of treatment and 1 week later were negative for helminth eggs. The goats were each infected with 5000 freshly cultured L_3 of a field strain of *Trichostrongylus colubriformis* on July 1999. Infective larvae required for conditioning were obtained by collecting faeces directly from goats over a week period, from July 24 to July 31. The faeces were cultured at 23 °C for 10 days. Infective larvae were recovered by Baermann extraction.

Design of experiments

Group 1: Each rabbit was given 5 x 150 freshly cultured L_3 every day (5 days).

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Group 2: Each rabbit received two doses of 750 freshly cultured L_3 at 5-day-interval.

Group 3: Each rabbit was infected with a single dose 10 000 freshly cultured L_3.

Group 4: Each rabbit was given 10 000 freshly cultured L_3. After 14 days were rabbits wormed and after further 8 days infected again with 750 freshly cultured L_3.

Control groups K1 (with immunosuppression): Each rabbit was infected with a single dose 750 freshly cultured L_3.

Control groups K2 (without immunosuppression): Each rabbit was infected with a single dose 10 000 freshly cultured L_3.

Control groups K3 (with immunosuppression): Non infected rabbits.

Control groups K4 (without immunosuppression): Non infected rabbits.

Immunosuppression of animals

Immunosuppression of animals were performed with continual administrations of prednisolone (Prednison 80 mg per rabbit and day). Start of immunosuppression was one week before inoculation L_3 to rabbits and end of immunosuppression was 3 days before killing of rabbits.

The immunosuppression of the rabbits with continuous administration of prednisolone showed no significant increase in the propensity of *Trichostrongylus* larvae for arrested development (S c h m i d , 1986). The immunosuppression of animals were performed because of prednisolone application in the first part of this experiment (L a n g r o v á , J a n k o v s k á , 2002) and for keeping comparable conditions of both parts of this experiment.

Immunosuppressed were all rabbits except control groups KB2 and KB4. Effect of rabbit immunosuppression was verified by means of the haematological parameters with apparatus Coulter CBC-5.

Recovery of worms

The rabbits were necropsied 4 weeks after infection. Gastric and intestine ingesta were decanted and larvae and adult parasites were counted. The larvae inhibited in development were released from the wall of stomach and intestine by means of digestion of mucosa in the solution of pepsin and hydrochloric acid (13 g pepsin, 10.8 ml HCl in 1000 ml of distilled water) for 4 hrs at 39 °C. Parasites were recovered by vigorously rubbing and washing the intestinal mucosae several times in water. The solution was decanted overnight and larvae were counted.

Statistical analysis

Due to binomic distribution the effect of factors was analysed by weighted logistic regression within generalised linear models (GLM). In each case, the mean percentage composition of arrested larvae recorded in the various treatment groups were compared with those recovered from control rabbits and examined for statistically significant differences by the chi-square test. In addition, the least significant difference (LSD) at the 1%, 5% and 10% level was calculated (C r a w l e y , 1993).

RESULTS

The present experiments were carried out in order to investigate the effect of the size of single and multiple infections upon the course of *Trichostrongylus colubriformis* infection in rabbits. The number of adult worms and EL_3 recovered from each of groups of the rabbits infected with *T. colubriformis* L are presented in Table 1.

The multiple infections with the low inoculation dose 150 *Trichostrongylus colubriformis* infective larvae every day (5 days) resulted in no inhibited larvae in intestine of rabbits (group 1). The repetition of infection with 750 *Trichostrongylus colubriformis* infective larvae after 5 days (2 x 750 L) led to 25.3% inhibited larvae in intestine of rabbits (group 2).

The high infection with 10 000 infective larvae induced 5.8% inhibited larvae in intestine of rabbits (Table 1). The repetition of infection after worming brought about 1.5% inhibited larvae (group 4 received the dose 10 000 L and after 14 days were rabbits of this group wormed. 8 days after worming animals received the second dose of 750 L).

Control group K1: Immunosuppresed rabbits infected with 750 infective larvae produced 5.28% inhibited lar-

Table 1. Numbers of *T. colubriformis* recovered from rabbits infected 20 days previously with multiple or single infection with freshly harvested viable infective larvae

Group	Nature of host	Larval dose	Nature of infective larvae	Worm counts				Mean EL_3 (%)
				total	adults	L4	EL_3	
1B	–	5 x 150	–	194	194	0	0	0
2B	–	2 x 750	–	247	179	1	67	25.3
3B	–	10 000	–	1989	1858	40	91	5.8
4B	treatment	10 000/750	–	306	304	0	2	1.5
KB1	immuno-suppression	750	–	678	588	19	71	5.3
KB2	–	10 000	–	2155	1761	0	394	18.1

EL_3 = exsheathed L_3

Table 2. Comparison of relative frequencies (F) of L_3 in experimental groups

Group	N	F	1B	2B	3B	4B	KB1	KB2
1B	194	0.0000		NS	NS	NS	NS	NS
2B	247	0.2526			NS	NS	$	NS
3B	1989	0.0577				NS	NS	#
4B	306	0.0147					NS	#
KB1	678	0.0528						#
KB2	2155	0.1809						

The frequencies were mutually compared using the least significant difference (LSD) test: $P < 0.1$ (#) and $P < 0.05$ ($)
N = number of EL_3 recovered from all rabbits in a group, NS = non significant difference

vae in intestine of rabbits. Group 2 (2 x 750 L) have significantly higher proportion of hypobiotic larvae ($P < 0.05$, Table 2) from group K1 (1 x 750 L_3).

Inoculation of rabbits with 10 000 infective larvae (without immunosuppression) caused 18.09% inhibited larvae in intestine of rabbits (control group K2). It was significantly higher proportion of hypobiotic larvae ($P < 0.05$, Table 2) from group 3 (1 x 10 000 L with immunosuppression), 4 (10 000 L / 750 L_3), K1 (1 x 750 L_3 with immunosuppression) (Fig. 1).

DISCUSSION

Inhibited development is a complex phenomenon, which is not completely understood and in gastrointestinal nematodes of ruminants seasonal climatic conditions and host resistance are considered to be the most important factors involved. The finding of a high degree of hypobiosis in non-immune tracer animals in many studies suggests that environmental factors rather than immunological responses may regulate the phenomenon (Armour et al., 1969a, b; Eysker, 1997).

The first experiments carried out by other authors indicated the earlier experience with parasite was useful for the inhibition. More worms (*T. retortaeformis*) appear to be arrested in previously infected than in susceptible rabbits. A greater proportion of a large dose of larvae is arrested than of a small dose (Michel, 1952b). That the arrested larvae resume their development in batches suggest a regulatory mechanism whereby the presence or absence of adult worms controls resumed development (Michel, 1974).

Also, the size of the infective larval dose has been associated with inhibition of development of *Obeliscoides cuniculi* (Rusell et al., 1966), *Ostertagia ostertagi* and *Cooperia oncophora* (Michel et al., 1975). A greater proportion of a large dose of larvae is arrested than of small dose (Michel et al., 1975). Massive infections provide a high degree of antigenic stimulus, thus the animal would develop a strong initial immune response (Fox, 1976). Michel (1963) interpreted that the excess larvae of *Ostertagia ostertagi* remain in the state of temporarily arrested development awaiting until some or all of the adult population dies or is eliminated by the host. Schmid (1986) also reported that the arrested development is influenced by the level of infection. *Obeliscoides cuniculi* infections initiated with 100 000 or more infective larvae were comprised of worm populations which were more than 50% inhibited larvae (Fox, 1976). However in this experiment the higher larvaeinoculum (10 000 infective larvae) led to

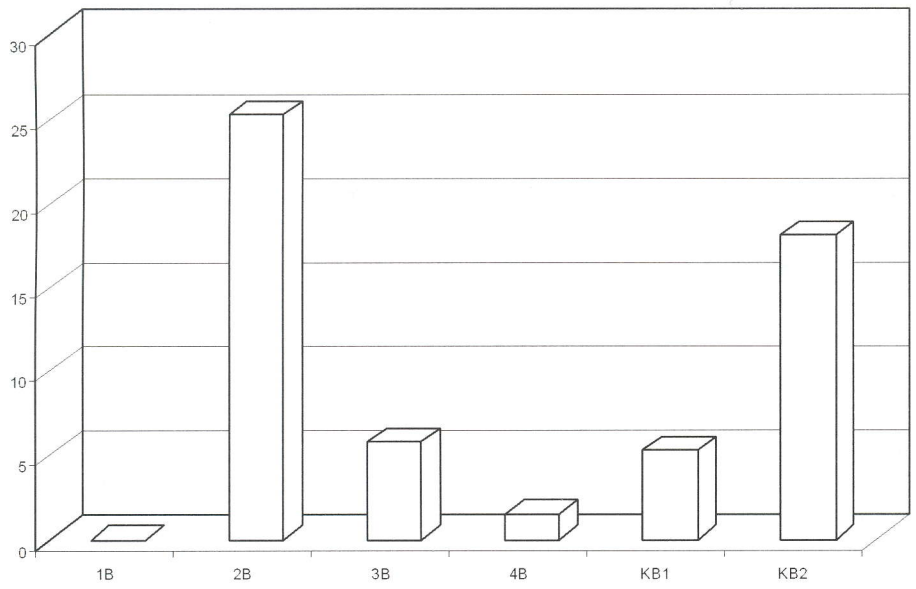

Fig. 1. The effect of acquired resistance, single, multiple doses and size of doses on rise of hypobiosis

x-axis – type of doses, y-axis – % hypobiotic larvae

5.77% of stages with arrested development only in group 3B (with immunosuppression) and to 18.09% of stages with arrested development in group KB2 (without immunosuppression). Also Fernando et al. (1971) have shown that the size of the inoculum of *Obeliscoides cuniculi*, at least in the range of 200 to 6000 larvae, had not appreciable effect on the level of arrested development which also corroborates the observations of Armour et al. (1969a, b) with *Ostertagia ostertagi* and of Dineen, Wagland (1966) and Smeal et al. (1980) concerning *Haemonchus contortus*.

Theory about association of hypobiosis with acquisition of immunity was later questioned since hypobiosis of *Ostertagia ostertagi* was found to be independent of the length of time grazed and previous infections (Armour et al., 1969a, b). Anderson (1965) postulated that inhibition of *Ostertagia ostertagi* was associated with physiological changes in the parasite, or possibly in the host, in late autumn and these changes were independent of the immunological status of the host.

The non-immune inhibition in nematode larvae has been reviewed by Armour, Bruce (1974), Michel (1974), Eysker (1993, 1997) and as cited this phenomenon could be ascribed to inherent developmental changes in the infective larval stages, either genetically or environmentally induced (Armour et al., 1969a, b; Eysker, 1997).

Records of arrested development of *Trichostrongylus* spp. are few. According to Michel (1952a), *Trichostrongylus retortaeformis* is arrested at the third stage and a large proportion of the larvae given to previously infected rabbits may fail to develop beyond this stage. In susceptible rabbits a very much smaller proportion of the worms is arrested. This study is a continuation of the first paper "Arrested development of *Trichostrongylus colubriformis* in experimentally infected rabbits. The effect of decreasing photoperiod, low temperature and desiccation (Langrová, Jankovská, 2002), which suggests that an environmental factor rather than immunological responses may regulate the hypobiosis.

The earlier experience with the parasite resulted in the present experiment in 25.26% of stages with arrested development at a dose 2 x 750 infective larvae at 5 days interval (group 2), however multiple infection with low dose 5 x 150 infective larvae every day led to no stages with arrested development. Small numbers of larvae probably do not elicit sufficient immune response to inhibit worm development. In group 4 rabbits were infected after deworming and it resulted in 1.47% of stage with arrested development only.

CONCLUSIONS

In conclusion, although the experiments reported here have not completely clarified the mechanism involved in inhibited development of *Trichostrongylus colubriformis* in rabbits and further study of the interaction between host and larvae is required, it is clear that a fundamental change taking place in the larvae exposed autumnal conditions is a primary requirement for inhibited development. It proved also our first work (Langrová, Jankovská, 2002).

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Pozastavení endogenního vývoje hlístice *Trichostrongylus colubriformis* v experimentálně nakažených králících. Vliv získané rezistence, jednorázové nebo opakované dávky infekčních larev *Trichostrongylus colubriformis* a velikost této dávky na vznik hypobiózy.

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V práci je popsán vliv opakování a velikosti infekční dávky na pozastavení endogenního vývoje (hypobiózy) vlasovky kozí (*Trichostrongylus colubriformis*) v modelovém hostiteli králíku domácím (*Oryctolagus cuniculus*). Na skupině 40 králíků bylo testováno ovlivnění endogenního vývoje parazita hostitelem. Byl zkoušen vliv velikosti a opakování infekční dávky. Pro pozastavení endogenního vývoje byla nejvýznamnější opakovaná dávka 750 L_3 (larvy 3. vývojového stádia hlístice *Trichostrongylus colubriformis*) po pěti dnech, kdy bylo zaznamenáno 25,26 % hypobiovaných larev, což je statisticky významný rozdíl vůči kontrole. U skupiny denně infikované dávkou 150 L_3 nebyly hypobiované larvy nalezeny vůbec. Podobně tomu bylo u skupiny 4B, kde byli králíci infikováni dávkou 10 000 L_3, následně odčerveni a znovu infikováni dávkou 750 L_3. V této skupině bylo pouze 1,47 % hlístic v hypobiované podobě. U skupiny 3B, kde byli králíci infikováni velkou inokulační dávkou (10 000 L_3), byl podíl hypobiovaných larev 5,77 %. Procento hypobiovaných larev bylo u této skupiny (3B) významně nižší než u kontrolní skupiny KB2, kde králíci také dostali 10 000 L_3, avšak bez podání imunosupresiva. V této skupině (KB2) bylo nalezeno 18,09 % hypobiovaných larev.

hypobióza; vlasovka kozí (*Trichostrongylus colubriformis*); modelový hostitel; králík domácí (*Oryctolagus cuniculus*)

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