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Requirement for Two Discrete Stimuli for Induction of the Intestinal Rapid Expulsion Response Against *Trichinella spiralis* in Rats

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Rats subjected to a 7-day abbreviated enteral infection with *Trichinella spiralis* subsequently reject more than 90% of a challenge infection within 24 h. This process is known as rapid expulsion. In these experiments parabiotic rats were used to examine the factors that establish rapid expulsion in the intestine. Induction with low to moderate doses of worms required exposure to two separate stimuli. These initiated different responses; one was readily transferred between parabiotic rats, whereas the second response was sessile and restricted to the intestine. These two responses interacted synergistically to produce strong rapid expulsion. Stage-specific exposure of parabiotic rats to preadult or adult trichinae (or the unrelated parasite *Heligmosomoides polygyrus*) showed that only preadult worms induced the transferable factor. Exposure to adult worms or to *H. polygyrus* induced a strictly local intestinal effect that was nonspecific. It is suggested that preadult worms initiated an immune response specific for preadults. This was transferable between parabionts but was unable to produce rapid expulsion unless the intestine had been non-specifically stimulated. Intestinal stimulation is accomplished by exposure to adult worms in natural infections or artificial regimes. These results suggest novel techniques for the development of enteral antihelminth vaccines.

Intestine-dwelling nematodes infect an estimated 10^9 humans (18, 20) and virtually all livestock. Despite their importance in human ecology and agricultural management, the basic mechanisms of acquired resistance to intestinal nematodes are poorly understood. *Trichinella spiralis* is a nematode naturally parasitic in carnivores, rodents, swine, and humans. Recent studies (4) have disclosed that immunized rats mount at least four distinct responses which mediate intestinal immunity against this parasite. One of these responses is expressed in the rapid expulsion (RE) (within 24 h) of 90% or more of a challenge infection with *Trichinella* larvae. RE is thus revealed as the principal arm of host defense against reinfection; yet the phenomenon has attracted little attention since it was described first by McCoy (13) nearly 40 years ago and more recently by a group of independent investigators (1, 2, 11, 15, 19).

Reactions operationally characterized by the prompt elimination from the intestine of parasitic nematodes have been described in sheep infected with *Haemonchus contortus* (17), in guinea pigs infected with *Trichostrongylus colubriformis* (14), in rabbits infected with *Trichostrongylus calcaratus* (16), as well as in rats infected with *Strongyloides rattii* (Bell, unpublished data) and in mice infected with either *Heligmosomoides polygyrus* (*Nematospiroides dubius*) (8) or *T. spiralis* (19). Although the kinetic similarity of these reactions and their expression in the gut suggest a common underlying mechanism, formal evidence to substantiate this will require basic information about the RE process.

The host-parasite relationship in *Trichinella*-infected rats affords an opportunity to analyze events associated with the induction and expression of RE. In this host species, RE is induced by a complete infection involving all stages of the life cycle or by exposure to either muscle-stage larvae or the enteral phases alone (4). More detailed analysis of the induction process has shown that infections limited to the preadult phase (first 30 to 36 h of enteral development) induce RE only after large or repeated infections. Likewise, large numbers of adult worms are required to induce RE (2). Since preadults and adults are very effective inducers of RE when combined (as in natural infections), it appears that these two phases induce separate, but synergistically interacting responses (1). Evidence that the response induced by the preadult

phase is immunological in nature comes from data showing that RE in rats is active only against preadult worms; adults are not susceptible (2). Furthermore, crude antigenic extracts of preadult *T. spiralis* can induce RE under certain conditions (3).

This paper analyzes the inductive requirements for RE in *Trichinella*-infected rats. Parabiosis was used because efforts to transfer RE reactions passively with serum were not successful and successful transfer with cells was difficult to reproduce (Bell, unpublished data). The experiments described below demonstrate the separate inductive roles of different phases of the *T. spiralis* enteral developmental process. By showing that transfer of reactivity between parabionts follows appropriate priming of the passive (nonimmunized) partner, the experiments demonstrate that two distinct responses are required for the expression of this intestinal defense in rats.

MATERIALS AND METHODS

Animals. (i) Rats. Male rats (weight, 175 to 300 g) belonging to the F_1 hybrid cross between either the Lewis and BN strains (LBN) (Microbiological Associates Walkersville, Md.) or the Lewis and DA strains (LDA) (Trudeau Institute, Saranac Lake, N.Y.) were used. Retired breeders of Fischer strain F344 (Microbiological Associates) were used as donors of larvae and adult (4-day-old) worms.

(ii) Parasites. (a) *T. spiralis*. *T. spiralis* was maintained by serial passage in retired breeder rats (Fischer strain). Infectious muscle larvae were obtained by pepsin hydrochloride digestion (37°C, 1 h of incubation) of minced rat carcasses. To isolate larvae, the digestion fluid was poured through cheesecloth to remove bones and undigested material and then through a 200-mesh sieve, which retained the larvae. These were washed off, counted, and diluted so that the required infectious dose was contained in 1 ml. Larvae were given to lightly anesthetized rats through a feeding tube.

(b) *Heligmosomoides polygyrus*. *H. polygyrus* was maintained by serial passage in CFW mice. Rats were infected orally with infectious larvae obtained from 6- to 7-day fecal cultures from infected mice. Rodents such as voles and mice are the natural hosts of *H. polygyrus*; in rats, the parasite invades the mucosa of the upper one-half to one-third of the small intestine (the same site as *T. spiralis*). Here it encysts and begins development in a manner comparable to its early life cycle in mice (7). In normal rats, the parasite usually fails to escape from the intestinal cyst as it does in mice. Therefore, its life cycle is interrupted at this point. Apparently, the strong connective tissue reaction mounted by rats around the cyst destroys the developing worms (7). The parasite thus provokes a strong intestinal reaction in rats.

Stage-specific immunization of rats. (i) Preadult immunization. Rats were infected orally with either 2,000 or 3,000 larvae. After 24 and 48 h each rat was injected subcutaneously with methyridine (300 mg/kg). This regime eliminated worms from the small intestines (4). The period of intestinal development from oral challenge (with larvae) until worm maturation to adulthood (approximately 33 h) (10) is referred to as the preadult stage. Although these worms are still larval, the use of the term preadult avoids confusion with either the infectious muscle larvae stage or the later newborn larvae stage produced by adult females in the intestines.

(ii) Adult immunization. Rats were immunized with adult *T. spiralis*; care was taken to avoid exposure of the hosts to the preadult stage of the parasite. Groups of retired breeder rats were infected with 5,000 to 9,000 muscle larvae and killed 4 days later. The small intestines were removed, slit open, and washed. They were then incubated at 37°C for 1 h in M199 medium containing 1,000 U of penicillin per ml. Thereafter, the intestines were discarded, and worms which had migrated into the culture medium were collected by sedimentation at unit gravity. After counting, the concentration of worms was adjusted so that the desired number was contained in 1 ml of medium. These were injected directly into the upper 2 cm of the small intestine through a midline laparotomy incision. Infected rats were immediately fed diets containing 0.05% thiabendazole (Merck & Co., Rahway, N.J.) to prevent newborn larvae production. At 10 and 11 days after transfer, methyridine (300 mg/kg) was injected subcutaneously to terminate the infection.

Standard RE regime. Rats were infected orally with 2,000 larvae. After 3 days the animals were given a diet containing 0.05% thabendazole to prevent newborn larvae production (4). At 7 and 8 days after infection, the rats were injected subcutaneously with methyridine to terminate the infection. This procedure is referred to below as the TM regime (thiabendazole/methyridine). In rats, the TM regime induced an RE response which persisted for approximately 5 to 6 weeks after the primary infection (1). Challenge infections were usually given 14 to 21 days after the primary infection. It should be noted that the TM regime is not phase specific, since the host is exposed to both preadult and adult stages of the enteral life cycle of the parasite.

Parabiosis of rats. LBN or LDA rats were joined side to side with anastomosis of the peritoneal cavities and union of muscles and skin from the scapula to the femur. At 3 weeks after surgery the establishment of an efficient cross-circulation was confirmed by infusing ^{51}Cr-labeled syngeneic erythrocytes into one partner of each parabiotic pair. Blood samples were drawn from both rats 2 h after injection. Radioactivity levels in the uninjected rats $\geq$80% of those in the injected rats were taken as evidence of efficient cross-circulation.

Assays of protection. The protection afforded by an immunizing infection was assessed by comparing the number of worms in the intestines of immune and nonimmune rats of the same strain. Measurements of RE were made 24 h after challenge; other manifestations of acquired resistance were evaluated 7 days later.

Statistical analysis. Experimental groups were compared by using Students *t* test for independent means. Probability levels of >0.05 were considered significant.

RESULTS

Transfer of RE through a shared circulation. This experiment was designed to determine whether the induction of RE in one rat led to the expression of RE in both that rat and a partner whose circulation was shared. In other words, could the reaction be transferred between parabiotic rats via the bloodstream? To test this question, rats were united surgically. After 5 weeks and after determining that an efficient cross-circulation was established in each pair, one partner (right) was infected with 2,000 *T. spiralis* larvae (Fig. 1). After 3 days all rats were fed 0.05% thiabendazole, and then at 7 and 8 days methyridine (300 mg/kg) was injected into both partners to terminate the infection. At day 14 all rats were challenged orally with 700 infectious *T. spiralis* larvae. A group of six parabionts and five challenged controls were harvested 24 h later to assay for RE. A second group of four parabionts and five controls were killed at 7 days to assay for slow rejection, which is characteristic of anti-preadult and anti-adult immunity (4).

The results (Fig. 1, group A) showed that RE was strongly expressed in the actively immune (right) partner but not in the uninfected (left) partner of the parabiotic pair ($P < 0.05$). When the rats were assayed 7 days after challenge, no worms were found in the actively immune parabionts (right), and a mean of two worms was found in the unimmunized (left) partners (Fig. 1, group C). The values for all rats in group C were significantly lower than those for the nonimmune controls (group D) ($P < 0.005$).

Transfer of RE between parabiotic rats after intestinal priming. The experiment described above showed that RE was not transferred from immune to nonimmune parabionts. These data suggested that a second factor was involved in the RE process. This unspecified factor was thought to involve a nonspecific change in the intestine. If this assumption were valid, then stimulation with an unrelated intestinal parasite might induce similar changes and thereby promote the expression of RE in appropriately immunized hosts. This was tested by priming the intestines of one member of each of five parabiotic pairs with 1,000 *H. polygyrus*. The partner of each of these *H. polygyrus*-stimulated rats was immunized concurrently with the TM regime, as described above.

Figure 2 shows that RE was not transferred when only one partner was immunized with *T. spiralis* (Fig. 2, group A), confirming the results of the first experiment (Fig. 1). Similarly, infection with *H. polygyrus* (Fig. 2, group B) did not cause RE by itself, nor did *H. polygyrus*-infected rats transfer RE to their partners. However, when the partners of rats immune to *T. spiralis* were infected with *H. polygyrus*, RE was expressed in both rats.

Relative importance of intestinal priming in the expression of RE. Earlier experiments had shown that a specific stimulus for RE was provided by larval and preadult *T. spiralis* and that immunizations with large numbers of larvae alone induced RE (2). To analyze the

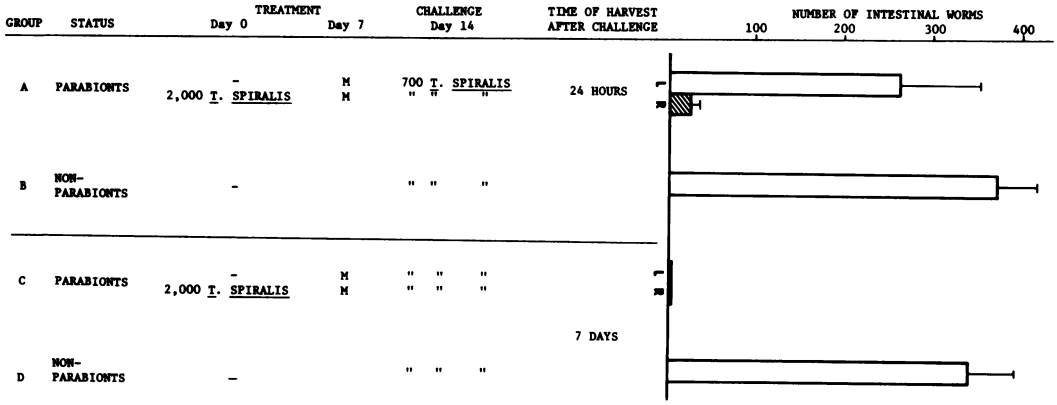

FIG. 1. *Ineffective transfer of RE between parabionts despite the presence of strong immunity in both partners. One partner (right [R]) in each of 10 parabiotic pairs was infected with 2,000 T. spiralis for 7 days (see text). This involved exposure to both preadult and adult worms. At 14 days after infection the parabionts and nonimmune controls were challenged. After an additional 24 h RE was evident in each actively immune parabiont (right rat) but not in the left (L) partner. By 7 days after challenge both parabionts had almost eliminated the challenge infection, thus demonstrating strong immunity in the nonimmunized (left) parabionts. M, Methyridine.*

contribution of the larva-preadult component relative to the intestinal priming component in the induction of RE, the following experiment was performed.

One partner (Fig. 3, group A, left rat) of five parabiotic pairs was infected on day 0 with 3,000 larval *T. spiralis*. The infection was restricted to preadult worms by treating all group A parabionts with methyridine (300 mg/kg) on days 1 and 2. Two non-parabiotic control groups (groups B and D) were treated identically. On day 4, the noninfected parabionts in group A (right partner) were infected orally with 500 *H. polygyrus*; another control group (group C) and group D were also infected. Group D was included as a positive control to demonstrate the effects in individual rats of a larval *T. spiralis* infection and a subsequent *H. polygyrus* infection on induction of RE. All groups were challenged with 1,000 *T. spiralis* 14 days after the *H. polygyrus* infection and were assayed for intestinal worms 24 h later. The results showed RE only in the parabiotic partners receiving 500 *H. polygyrus* (group A, right rat) and in the rats

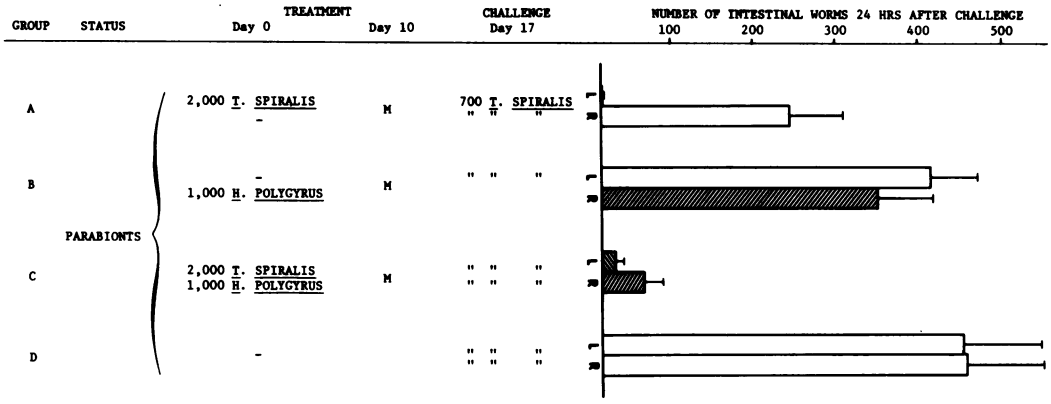

FIG. 2. *Transfer of RE between parabionts after intestinal stimulation. In the four parabiotic groups, T. spiralis immunization with the TM regime (preadults and adults) occurred in the left (L) partner only of group A. In group B, H. polygyrus stimulation occurred in the right (R) partner only. Group C combined T. spiralis stimulation (TM regime) of the left partner with H. polygyrus stimulation of the right partner. Group D provided a challenge control. After challenge, RE occurred in both parabionts only if both T. spiralis exposure and intestinal stimulation with H. polygyrus took place (group C). M, Methyridine.*

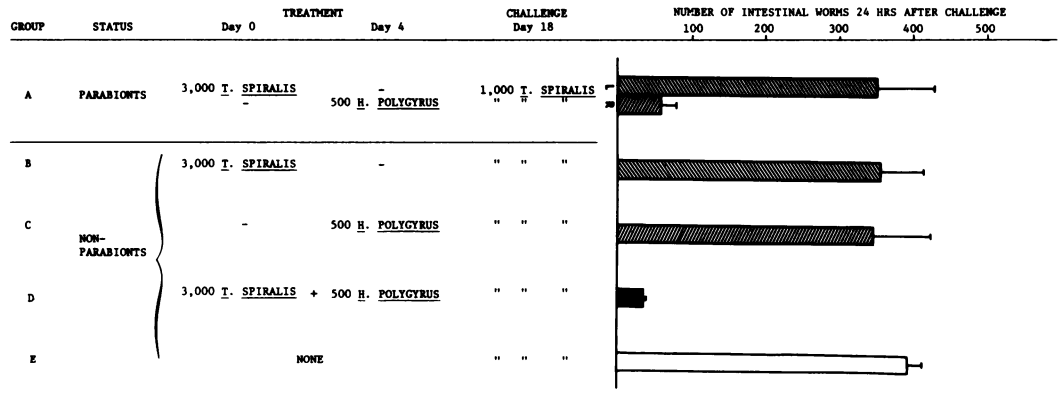

FIG. 3. *Transfer of RE to intestinally primed parabionts after exposure limited to preadult T. spiralis in actively immune rats. Within the parabiotic group (group A) the left (L) rats were immunized with preadult worms only (see text). The right (R) rats were infected with 500 H. polygyrus on day 4. Groups B through E provided the appropriate immunization and infection controls in single rats. At 14 days after infection with H. polygyrus, all rats were challenged with 1,000 muscle larvae and assayed 24 h later. In the parabiotic pairs (group A), RE was expressed only in the intestinally stimulated (right) rats. Preadult exposure alone (left rats) did not produce RE.*

infected with both *H. polygyrus* and preadult *T. spiralis* (group D).

Role of preadult and adult worms in the induction of RE. The next experiment was identical in plan with the above-described experiment except that adult (4-day-old) *T. spiralis* worms were used instead of *H. polygyrus* in one of the partner rats. This design was intended to define the roles of *Trichinella* preadults and adults in the induction of RE and particularly to determine whether adult worms primed the intestine.

Figure 4 shows RE in group D, demonstrating the strong inductive properties of consecutive exposure to preadult and then adult worms in single rats. A statistically identical RE was also observed in the parabionts that received 500 adult worms alone (also significantly different from either group B or group C [$P < 0.005$]). In contrast, the parabiotic partners exposed to 3,000 preadult worms showed only weak RE, as did control groups B and C, which were exposed to either preadult or adult worms alone. This degree of reactivity in the control groups most likely represented a higher than usual infectivity of both preadult and adult worms. Notwithstanding this effect, it is significant that full RE occurred only in the parabionts exposed to adult worms.

Character of the response to adult worms. Earlier experiments (2) had shown that heavy infections (with 2,000 or more adult worms) induced RE in the absence of preadult worm exposure. Several aspects of this response to adult infection suggested that it was a nonspecific intestinal reaction to the heavy adult worm burden. However, we could not discount the possibility that the relevant antigens of *T. spiralis* larvae were secreted in small quantities or expressed by adult worms, leading to sensitization of rats heavily infected with adult worms. The possible sensitization of rats to preadult antigens by exposure to large numbers of adults was examined in an experiment in which one rat (left partner) of a parabiotic pair was infected with 4,000 adult worms, a level which induced RE in that rat. The right partner was infected with 400 *H. polygyrus* to prime the intestine. It was anticipated that any immunologically specific factor generated by the heavy infection with adults would be transferred to the intestinally primed (*H. polygyrus*) partner to induce RE capabilities. This experiment duplicates the one shown in Fig. 3, group A, except that a large number of adult worms were substituted for preadult exposure. The results show that RE occurred in rats given only 4,000 adult worms (Table 1, groups B and A, left partners), whereas treatment with 400 *H. polygyrus* did not produce RE.

DISCUSSION

Parabiotic rats have been used to examine the immune response to *T. spiralis* before (21–23). The pioneering work of Zaiman showed that immunity generated in *Trichinella*-infected rats can be transferred to uninfected parabiotic partners. RE was never demonstrated in these experiments, undoubtedly because the need for intestinal priming was not recognized. However, other technical difficulties cloud the interpretation of the studies of Zaiman. For example, randomly bred rats were used, and no attempt was made to examine the cross-circulation between parabiotic pairs; in several experiments larvae were not restricted to the muscles of the infected partners. These deficiencies in design were avoided in the current investigation, as inbred rats were used and the presence of an efficient cross-circulation was established before

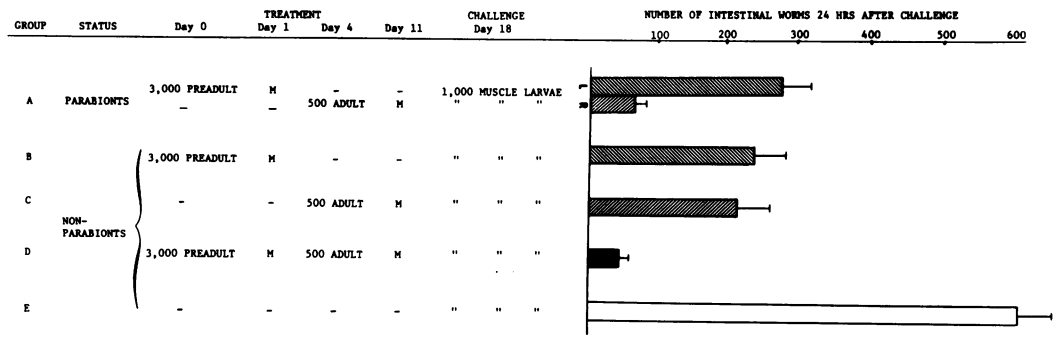

FIG. 4. *Intestinal stimulation induced by exposure to adult T. spiralis worms. This experiment shows the transfer of a factor from preadult T. spiralis-stimulated rats (group A, left [L] rats) to rats that had been primed with adult T. spiralis. The protocol was as described in the legend to Fig. 3. R, Right rats; M, Methyridine.*

the use of any parabiotic pair. Treatment with the antihelminthics thiabendazole and methyridine ensured restriction of the parasite to the intestines of infected partners.

The results show that two factors are required for the expression of RE in rats immunized by exposure to moderate numbers of *T. spiralis*. One factor is generated in response to preadult worms or their products; the other involves a priming effect on the intestine.

The first experiment (Fig. 1) showed that the induction of vigorous RE in a parabiotic rat after exposure to the TM regime did not result in the transfer of RE to its normal partner. Although RE was not transferred, slow adult worm rejection (occurring from days 5 to 8 after challenge) was transferred effectively. This response is characteristic of the immunity induced by and expressed against the preadult and adult stages of the life cycle and may be taken as evidence of strong immunity (4). Therefore, the presence of active immunity is not in itself sufficient to cause RE. These results are essentially identical to those of Zaiman et al. (21–23) and are open to the interpretation that RE is a local phenomenon which lacks an immunological basis.

Earlier data had suggested that a local component was important in the induction of RE (Bell, unpublished data), and therefore the experiment was repeated with the modification that the nonimmune partner was given a priming infection with *H. polygyrus* to stimulate the intestine. Under these conditions RE was expressed with equal vigor in both the *T. spiralis*- and *H. polygyrus*-infected parabionts (Fig. 2). Since parabionts exposed to *H. polygyrus* alone did not show RE, it was apparent that the RE response required both *H. polygyrus* intestinal stimulation and some other factor which was (presumably) transferred from the *T. spiralis*-infected rats.

Evidence previously accumulated had shown that in rats RE is specific for preadult worms as opposed to adult worms, although preadult worms by themselves have a limited capacity to induce the response (2). The interaction of the adult and preadult stages, as it takes place in the intestine during normal worm development, is crucial to the development of RE. Since the parabionts were ideally suited to an examination of the interactions of each of the intestinal stages of development, we extended our experiments to examine the inductive properties of each stage separately.

In doing this, we accepted that intestinal stimulation was essential for the expression of RE, and in the next experiment we focused on the role of preadult *Trichinella* (Fig. 3). In this experiment exposure limited either to preadult worms or to *H. polygyrus* had no potential to induce RE, although rats infected with *T. spiralis* first and then *H. polygyrus* showed strong RE (Fig. 3, group D). In the parabiotic pairs (group A) in which *T. spiralis* preadults were given to one rat and *H. polygyrus* was given to the other, RE was strongly expressed in the rats receiving *H. polygyrus*. RE was thus only expressed at the site of intestinal stimulation, and an effect was not observed in the absence of exposure to preadult *Trichinella*. Intestinal stimulation is thus a sessile phenomenon which cannot be transferred but facilitates the expression of a factor(s) transferred from a rat primed with preadult *T. spiralis*. The intestinal effect and the transferred factor interact synergistically rather than additively in producing RE.

To confirm that the adult worms fulfilled the intestinal priming role, the above experiment was repeated by using adult *T. spiralis* instead of *H. polygyrus*. The results confirmed our supposition that adult worms primed the intestines and showed that RE again occurred only in the intestines primed with adults (Fig. 4). This experiment therefore supported the data shown in Fig. 3, indicating that RE occurs at the site of intestinal stimulation. An additional experiment was performed to analyze the possibility that large numbers of adult worms could induce the transferable component of the response (normally a function of preadults). Since they were

TABLE 1. *Failure to transfer RE between parabionts after infection with high doses of adult T. spiralis in one partner*

Group	Rat	Status	Treatment	Day of challenge	No. of intestinal worms[a]
A	Right	Parabionts	400 *H. polygyrus*	17	308 ± 162
	Left	Parabionts	4,000 adult *T. spiralis*[b]	17	63 ± 65
B		Nonparabionts	4,000 adult *T. spiralis*	17	22 ± 17
C		Nonparabionts	400 *H. polygyrus*	17	456 ± 207
D		Nonparabionts		17	672 ± 215

[a] Rats were harvested 24 h after challenge with 1,000 *T. spiralis* muscle larvae. In comparisons between group A, right rats, and group C and between group A, left rats, and group B, differences were not significant. In a comparison between group A, right rats, and group A, left rats, $P < 0.01$.

[b] Adult worms were transferred by laparotomy and direct injection into the intestine.

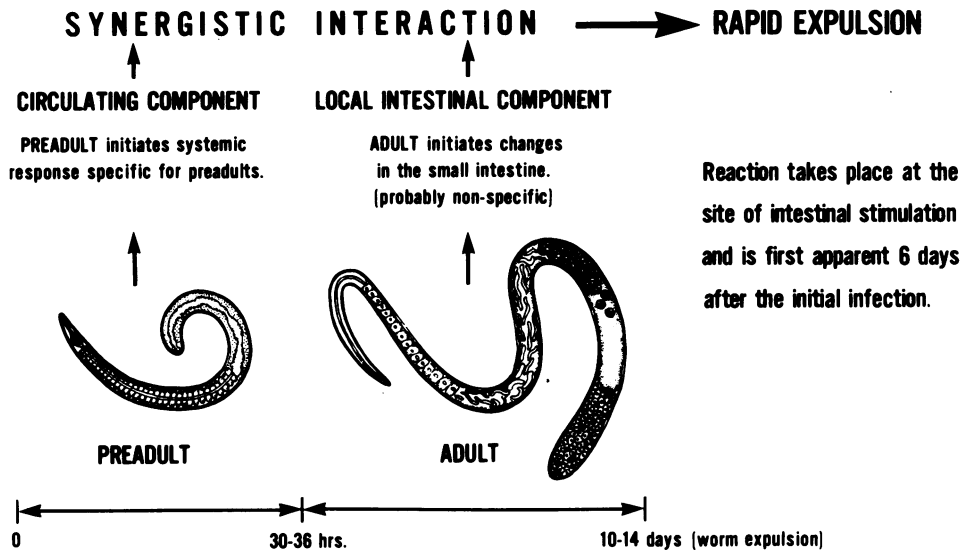

FIG. 5. *Induction of the intestinal rapid expulsion response: requirement for two priming stimuli. Diagrammatic representation of the interacting effects of preadult and adult Trichinella worms in intestines.*

not able to do this (Table 1), it was concluded that the adult worms act solely to prime the intestine, whereas the preadults stimulate a circulating factor which operates at the site of intestinal stimulation. This scheme is shown diagrammatically in Fig. 5, which shows the stage specificity of the overall response pattern.

The circulating factor(s) generated in response to preadult *T. spiralis* has not been identified. However, several lines of evidence suggest that it is either antibody or a specifically sensitized lymphocyte, or both. First, the factor concerned is generated in response to preadults but not adults. This observation accords with the suggestion that relevant antigens are expressed transiently during the preadult phase of the enteral development of the parasites (4). Second, RE can be induced by antigenic cell-free extracts of preadult *T. spiralis*, a finding which indicates that the inductive stimulus is not a peculiar property of living parasites (3). Third, the present study indicates that the capacity to express RE can be transferred from infected parabionts to uninfected partners. And finally, RE can be transferred to intestinally primed rats by injections of living cells obtained from the thoracic ducts of specifically immunized donors (Bell, unpublished data).

It is not known whether the factor(s) generated in response to preadult *T. spiralis* causes RE, amplifies this reaction, or triggers other events through which the expulsion process is ultimately expressed. It is evident, however, that the factor(s) concerned mediates RE only in animals whose intestines have been suitably primed. The absolute requirement for intestinal priming has not been recognized previously and may account for the low levels of immunity induced by artificial immunization in both rats and mice (5, 6, 9, 12). Since RE is the most effective protective response during a challenge infection, its absence after artificial immunization would severely limit the level of protection. Now that the interaction between preadult and adult *T. spiralis* is appreciated, it may be possible to devise immunization protocols that favor the expression of RE. It remains to be seen whether similar interactions occur in animals harboring other intestinal nematodes. If RE is an important defense against such nematodes and its mechanism is similar to that revealed in *Trichinella*-infected rats, it may be possible to utilize this knowledge in the development of effective immunization strategies. A preliminary step in this direction is reported in the accompanying paper (3).

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