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Objekttyp: **Article**

Zeitschrift: **Acta Tropica**

Band (Jahr): **36 (1979)**

Heft 4

PDF erstellt am: **26.09.2024**

Persistenter Link: https://doi.org/10.5169/seals-312534

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Immunization against *Nippostrongylus brasiliensis* in the rat

**A study on the use of antigen extracted from adult parasites
and the parameters which influence the level of protection**

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Summary

It was found that protective immunity in excess of 90% reduction in worm
burden could be stimulated against *Nippostrongylus brasiliensis* in rats by using
an extract of adult *Nippostrongylus* worms. The level of protection achieved was
influenced by several factors. Thus, the use of *Bordetella pertussis* as adjuvant
significantly increased the level of protection which, in addition, was shown to
be influenced by the amount of worm antigen used. Furthermore, antigen
administered in multiple doses was more effective than a single inoculum and,
when using such a regime, the interval between doses was also found to be
critical. The route of antigen administration was important and, while protec-
tion was achieved by subcutaneous and oral administration, the intraperitoneal
route was the most effective. Using the optimal immunization regime of 3 doses
of 5 mg worm protein and 4×10^{10} *B. pertussis* organisms, as adjuvant, levels of
protective immunity in excess of 90% reduction in worm burden were shown to
exist for at least 60 days after the last dose. It was found that adult worm extracts
did not stimulate any obvious immunity against larval forms of *N. brasiliensis*.

Key words: nematode; *Nippostrongylus brasiliensis;* adult worm extracts;
immunization; protection; worm burden; egg output; mast cells; reaginic an-
tibodies; *Bordetella pertussis;* adjuvant; dose of antigen; number of doses and
interval between; route of administration; memory.

Introduction

Despite the availability of effective drugs and of other control measures,
helminth disease remains one of the major health problems of man and his

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domestic animals. So far, only in the domestic animals are effective vaccines commercially available. These include vaccines against *Dictyocaulus viviparus* in cattle (Jarrett et al., 1960a) and *Dictyocaulus filaria* in sheep (Jovanović et al., 1965). Such vaccines employ as immunogen larvae attenuated by X-irradiation. A similar approach has been adopted with several other economically and socially important diseases. In some cases the results have been highly successful such as with *Ancylostoma caninum* in dogs (Miller, 1965) and with *Syngamus trachea* in chickens (Varga, 1968) while in other instances the level of protection obtained was promising but now awaits further development (reviewed by Urquhart et al., 1962).

The other approach to the development of helminth vaccines has been the use of killed worm antigens; these include worm homogenates, worm extracts and metabolic products. So far the results with non-living material have usually been considered equivocal and disappointing (Ogilvie and Jones, 1973), although there are a number of studies demonstrating significant levels of protection against nematodes, cestodes and trematodes (see discussion).

Recent advances in immunology have indicated that the host's immune response can be modulated to achieve a desired response and many of the variables involved in producing selective immunologic effects have been delineated. At the same time, there has been increasing understanding of the immunologic effector mechanisms operative in the helminth infections. Thus, there is a case for reconsidering the production of further helminth vaccines.

In the present study, using the model system of *Nippostrongylus brasiliensis* in the rat, ways of manipulating the host's protective response to killed adult worm antigen were examined with a view to achieving optimal protection. It was found that high levels of protection can be obtained using non-living parasitic material and that several factors can influence the outcome. These factors include the use of adjuvant, the dose of antigen, the number of doses given and the interval between them, and the route of administration.

Materials and methods

In nippostrongylosis, following systemic migration of infective larvae from a subcutaneous inoculation site, adult worms become established in the small intestine 5 days later. Following a stable phase, the parasites are expelled exponentially after day 10; this is preceded by a drop in worm egg output. The slope of the line showing the kinetics of worm expulsion is taken as a measure of the immune status of the host (Jarrett et al., 1968). Associated with worm expulsion there is an exponential increase in the number of intestinal mast cells and reaginic antibody levels in the serum become elevated (Miller and Jarrett, 1971; Murray, 1972). Thus, the parameters employed in the present study to judge the protective response to various vaccination regimes were worm burden, worm egg output, intestinal mast cell numbers and serum reaginic antibody response. Protection achieved was expressed as a percentage of the reduction in geometric mean worm number in the treated groups in comparison with the challenge controls.

Adult female hooded Lister rats aged 8 to 10 weeks and weighing approximately 150 g were used in all experiments. The parasite culture was prepared after the modified method of Bakarat (1951) adapted by Jennings et al. (1963). The technique of worm recovery from the small intestine

was described by Mulligan et al. (1965). Total worm counts were carried out in all cases in order to eliminate the sampling error caused by worm clumping. Faecal egg counts were done after the method of Gordon and Whitlock (1939).

Small intestinal tissues collected for mast cell quantitation were fixed and stained as described by Miller and Jarrett (1971). Intestinal mast cells were quantified on a villus-crypt (VC) unit basis in groups of 5 to 10 rats after the method of Miller and Jarrett (1971).

Serum from each group of rats was pooled and titrated for reaginic antibody activity by the passive cutaneous anaphylaxis test (PCA) performed in parasite-free rats. PCA was carried out by intradermal injection of doubling serial serum dilutions (0.1 ml), each dilution being duplicated in separate test animals. Seventy-two hours later, the rats were injected intravenously with 0.5 ml of *N. brasiliensis* antigen (containing 1 mg worm protein) in 0.5 ml of 1% Evans Blue. The rats were sacrificed 30 min later. The reagin titre was recorded as the reciprocal of the greatest dilution which gave skin reaction sizes of greater than 5 mm.

Adult worm antigen extract for use in vaccination studies and reaginic antibody estimations was prepared as follows. Rats were inoculated subcutaneously with 3000 to 5000 third stage *N. brasiliensis* larvae and were sacrificed 7 days later. The adult worms were harvested as described above and then washed repeatedly in phosphate buffered saline. The worms were then homogenised in an icebath using a Silverson Emulsifier (Silverson Machines Ltd.). The resultant homogenate was spun at 1700 g in a refrigerated centrifuge for 30 min to remove the larger worm fragments and eggs and then at 100,000 g for 50 min. The protein content of the resultant supernatant was estimated by the Lowry technique (Lowry et al., 1951) and then aliquots were stored at $-20°$ C. Throughout these procedures, particular attention was paid to maintaining worm homogenates in the cold.

The *Bordetella pertussis* (Wellcome Research Laboratories) used as adjuvant was a killed bacterial suspension containing 4×10^{10} organisms/ml. The standard dose employed in these studies was 4×10^{10} organisms given intraperitoneally.

All experiments used groups of 10 to 20 rats. The worm antigen and the adjuvant were administered intraperitoneally by separate inoculation but at the same time. Immunized rats were challenged subcutaneously with 3000 third stage *N. brasiliensis* larvae, unless otherwise stated, and were sacrificed 10 days later, just prior to the onset of worm expulsion in susceptible challenge control rats (Murray et al., 1971). Rats subjected to reinfection were inoculated with 3000 *N. brasiliensis* larvae at the start of the experiment and challenged with 3000 larvae at the same time as the vaccinated groups.

Prior to analysis, logarithmic transformation (to base 10) of worm burden and mast cell data was carried out. This transformation linearises the relationship between worm count or mast cell numbers and time where an exponential situation exists, as it does with *N. brasiliensis* (Jarrett et al. 1968; Miller and Jarrett, 1971), and normalises the data for statistical analysis. The studentised range test (Miller, 1966) was used to identify different groups. Values of P$<$0.05 were considered significant. All results in the text concerning worm counts and mast cell numbers are expressed as geometric means (GM) and standard error of the $\log_{10}$ values. In the tables, the results were given as geometric means and the pooled standard error of the $\log_{10}$ values, SE ($\log_{10}$).

Results

Several preliminary experiments established that significant levels of protection could be achieved against *N. brasiliensis* in the rat using whole adult worm extract, e.g., one group of 12 rats, which received 2 inoculations of 5 mg worm protein intraperitoneally at a 30 day interval and challenged 10 days later with 3000 *N. brasiliensis* larvae, was found to harbour 475 ± 0.15 worms 10 days later as compared with 968 ± 0.03 worms in the challenge controls, a significant 50% reduction in worm burden.

Table 1. Effect of the adjuvant *Bordetella pertussis* on worm burden, intestinal mast cells and reaginic antibody levels in rats immunized against *Nippostrongylus brasiliensis*

	Group				
	1	2	3	4	5
No. of rats	20	20	20	20	20
Preparation	Antigen/*B. pertussis*	Antigen	*B. pertussis*	Reinfection	Challenge controls
Vaccine regime					
dose in mg worm protein	5	5	–	–	–
no. of doses	3	3	3	–	–
dose interval in days	7	7	7	–	–
days to challenge	10	10	10	24	–
Worm burden, GM ± pooled SE ($\log_{10}$)	19 ± 0.17^b	243 ± 0.17^c	1157 ± 0.17^d	4 ± 0.17^a	1161 ± 0.17^d
% protection	98	79	0	99	–
Mast cells/VC, GM ± pooled SE($\log_{10}$)					
before*	11 ± 0.02^b	9 ± 0.02^a	9 ± 0.02^c	35 ± 0.02^c	9 ± 0.02^a
after	6 ± 0.13^b	5 ± 0.13^b	1 ± 0.13^a	70 ± 0.13^c	1 ± 0.13^a
Reaginic antibody titre	1/1024	1/16	0	1/1024	0

Different subscripts indicate different groups by the studentised range test at a 5% significance level.
* 5 rats were sacrificed from each group prior to challenge.

The following series of experiments examined several parameters which might affect levels of protection. These parameters included the influence of adjuvant, the importance of dose of antigen, comparison of the effect of single or multiple doses, the effect of the interval between doses, and the importance of the route of administration of antigen. In addition, the site of action of adult worm antigen was considered as was the duration of immunity.

Influence of adjuvant

B. pertussis was chosen as adjuvant because of its established ability to potentiate sensitivity to mast cell-mediated anaphylaxis (Mota, 1958), a response possibly important in immunity to *N. brasiliensis* (reviewed by Murray, 1972).

It was found that the simultaneous administration of *B. pertussis* with whole adult worm extracts significantly increased the level of protection obtained. Table 1 shows the results of a typical experiment and the experimental procedure. With worm antigen alone, the average level of protection was 79%. When *B. pertussis* was also administered the level of protection rose to 98%, a significant increase. This was almost of the same order found in rats immunized with live parasites (99%). *B. pertussis* alone had no protective effect. The results were reflected by higher levels of intestinal mast cells and reaginic antibodies and also by a quicker reduction in worm egg output in the protected groups (Table 2).

In a similar experiment using smaller amounts of worm protein, 2 mg per dose, comparable although slightly reduced levels of protection were obtained. The number of worms recovered from antigen/*B. pertussis* immunized rats was 113 ± 0.23, the antigen alone group contained 800 ± 0.4 worms, while the challenge controls harboured 1327 ± 0.03 worms. Thus, the level of protection rose significantly from 40% to 91%, simply by the additional use of the adjuvant *B. pertussis*. As in the previous experiment, these results were reflected by a quicker reduction in worm egg output and a significant increase in intestinal mast cells and serum reaginic antibody response in the protected groups.

Table 2. Effect of the adjuvant *Bordetella pertussis* on worm egg output/g faeces in rats immunized against *Nippostrongylus brasiliensis*

Group	Preparation	Days after challenge		
		8	9	10
1	Antigen/*B. pertussis*	24,900	1,500	0
2	Antigen	36,200	5,900	2,400
3	*B. pertussis*	38,100	30,000	17,100
4	Reinfection	600	150	0
5	Challenge controls	34,000	25,000	19,200

Table 3. Effect of antigen dose on worm burden, intestinal mast cells and reaginic antibody levels in rats immunized against *Nippostrongylus brasiliensis*

	Group			
	1	2	3	4
No. of rats	12	12	12	12
Preparation	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Challenge control
Vaccine regime:				
dose in mg worm protein	1	10	30	–
no. of doses	1	1	1	–
days to challenge	10	10	10	–
Worm burden, GM ± pooled SE($\log_{10}$)	740 ± 0.11[b]	630 ± 0.11[b]	77 ± 0.11[a]	991 ± 0.11[b]
% protection	25	36	92	–
Mast cells/VC, GM ± pooled SE($\log_{10}$)	2.2 ± 0.16[a]	6.5 ± 0.16[b]	11.2 ± 0.16[b]	1.0 ± 0.16[a]
Reaginic antibody titre	1/512	1/1024	1/1024	1/16

Different subscripts indicate different groups by the studentised range test at a 5% level.

Effect of antigen dose

The result of the above 2 experiments suggested that the dose of antigen administered might have influenced the level of protection achieved. In order to evaluate the importance of dose in relation to protection, three groups of rats were immunized with three different amounts of worm antigen. The results are shown in Table 3. It was found that there was a significant correlation between the dose of antigen used and the degree of the protection obtained. The average protection achieved by 1 mg, 10 mg and 30 mg worm protein was 25%, 36% and 92%, respectively, as judged by reduction in worm burden. As before, this was reflected by an increase in intestinal mast cells and by reaginic antibody titres. The worm egg output was markedly reduced in the most effectively protected group.

Single or multiple immunizing doses

In order to evaluate the potential influence of single or multiple doses on protection, 3 groups of rats were given a total of 15 mg worm protein on one, two or three separate occasions. Ten days after the last inoculum they were challenged with 2000 *N. brasiliensis* larvae (Table 4).

Significantly higher levels of protection (99%) were achieved when a triple dose was given at weekly intervals than when only one dose was administered (93%). When 2 doses were given at a 30 day interval significant protection was obtained but it was lower (71%) than with the other regimes. These results were again reflected by a more rapid drop in worm egg output and a significant increase in intestinal mast cells and reaginic antibody titres in protected rats.

Influence of the interval between immunizing doses

A possible explanation for the lower level of protection obtained in the previous experiment, when two doses of worm antigen were given at a 30 day interval, was that the interval between doses is critical and that 30 days was too long to obtain suitable priming and a good secondary response in this particular system. Thus, an experiment was carried out with 2 groups of 12 rats; one group received 2 doses of 7.5 mg worm protein at a 7 day interval while the other group was given the same amount at a 30 day interval. The adjuvant *B. pertussis* was used with each dose. The rats were challenged 10 days later with 3000 *N. brasiliensis* larvae. When sacrificed 10 days later the level of protection produced in the rats receiving the immunizing doses at a 7 day interval was 89% (worm burden = 116 ± 0.18), as judged by the percentage reduction in worms. This was significantly higher than in the group of rats which were immunized at a 30 day interval; the level of protection in this group was 61% (worm burden = 429 ± 0.14), confirming that the time interval between immunizing doses is a critical consideration in vaccine protocols. The number of worms recovered from the challenged controls was 1110 ± 0.03.

Table 4. Effect of multiple or single doses of worm antigen on worm burden, intestinal mast cells and reaginic antibody levels in rats immunized against *Nippostrongylus brasiliensis*

	Group			
	1	2	3	4
No. of rats	10	10	10	10
Preparation	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Challenge controls
Vaccine regime:				
dose in mg worm protein	5	15	7.5	—
no. of doses	3	1	2	—
dose interval in days	7	—	30	—
total dose in mg worm protein	15	15	15	—
days to challenge	10	10	10	—
Worm burden, GM±pooled SE($\log_{10}$)	3 ± 0.18[a]	37 ± 0.18[b]	163 ± 0.18[c]	568 ± 0.18[d]
% protection	99	93	71	—
Mast cells/VC, GM±pooled SE($\log_{10}$)	40 ± 0.1[c]	12 ± 0.1[b]	11 ± 0.1[b]	4 ± 0.1[a]
Reaginic antibody titre	1/1024	1/512	1/256	1/4

Different subscripts indicate different groups by the studentised range test at a 5% significance level.

Table 5. Effect of route of administration on worm burden, intestinal mast cells and reaginic antibody levels in rats immunized against *Nippostrongylus brasiliensis*

	Group			
	1	2	3	4
No. of rats	10	10	10	10
Preparation	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Challenge control
Vaccine regime				
route of administration of antigen	Intraperitoneal	Subcutaneous	Oral	—
dose in mg worm protein	10	10	10	—
No. of doses	2	2	2	—
dose interval in days	10	10	10	—
days to challenge	10	10	10	—
Worm burden, GM ± pooled SE(log_{10})	41 ± 0.1^a	229 ± 0.1^b	661 ± 0.1^c	1026 ± 0.1^c
% protection	96	77	35	—
Mast cells/VC, GM ± pooled SE(log_{10})	16.3 ± 0.11^b	14.5 ± 0.11^b	2.3 ± 0.11^a	1.9 ± 0.11^a
Reaginic antibody titre	1/1024	1/512	1/128	0

Different subscripts indicate different groups by the studentised range test at a 5% significance level

Table 6. Effect of time to challenge on worm burden, intestinal mast cells and reaginic antibody levels in rats immunized against *Nippostrongylus brasiliensis*

	Group			
	1	2	3	4
No. of rats	12	12	12	12
Preparation	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Challenge control
Vaccine regime				
dose in mg worm protein	5	5	5	–
no. of doses	3	3	3	–
dose interval in days	7	7	7	–
days to challenge	60	30	10	–
Worm burden, GM ± pooled SE($\log_{10}$)	15 ± 0.29[a]	9 ± 0.29[a]	28 ± 0.29[a]	1127 ± 0.29[b]
% protection	98	99	97	–
Mast cells/VC, GM ± pooled SE($\log_{10}$)	3 ± 0.12[b]	7 ± 0.12[c]	14 ± 0.12[c]	1 ± 0.12[a]
Reaginic antibody titre	1/2048	1/4096	1/4096	0

Different subscripts indicate different groups by the studentised range test at a 5% significance level.

Importance of route administration

In the present series of experiments, it was shown that the protective effect of antigen prepared from adult worms appears to be effective only when the parasites have matured to the adult stage and are in the small intestine (see later). Thus, it was decided to compare the efficacy of the local or oral route of administration with parenteral ones. It was found that the oral administration of antigen produced only low levels of protection (35%) and was much less effective than the parenteral routes, where the subcutaneous route (77%) was found to be inferior to the intraperitoneal one (96%) (Table 5).

As with other experiments, reaginic antibody titres and intestinal mast cell numbers were increased and correlated with the level of protection achieved.

Duration of immunity achieved using killed adult worm antigen

In order to examine the duration of immunological memory induced using killed worm antigen, three groups of rats were immunized using the vaccine procedure found to be optimal in the present study, namely, 3 doses of 5 mg worm protein inoculated intraperitoneally at intervals of 7 days. 4×10^{10} organisms of *B. pertussis* were given intraperitoneally with each dose. One group of rats was challenged 10 days, one at 30 days and another at 60 days after the last inoculation. It was found that high levels of immunity were maintained for at least 60 days (Table 6). Significantly increased levels of intestinal mast cells and elevated reaginic antibody titres corresponded with the protective responses achieved.

Site of action of killed adult worm vaccine

There was some evidence in the previous experiments, as judged by worm egg output, that following immunization and challenge adult worms became established in the small intestine of immunized rats. To examine this possibility and to define at what stage of the parasite life cycle this particular vaccine was operative, an experiment was carried out in which immunized rats were sacrificed on day 6 as well as day 10 after challenge. The results are shown in Table 7. On day 6 there was no difference in the number of parasites established in the small intestine of the vaccinated animals as compared with the challenge controls. By day 10, however, there was a highly significant reduction of over 90% in the vaccinated groups. This result indicated that the killed adult worm vaccine was effective only against the adult parasites and apparently showed no cross reactivity against migrating larval forms. In the protected rats, there was a corresponding increase in the number of intestinal mast cells and in the serum reaginic antibody levels.

The result obtained from the group of rats immunized with only one dose of antigen, while showing no significant difference from the group which received 3 doses, once again gave some indication that dose was an important factor in induction of protective immunity.

Table 7. Site of action of adult worm antigen vaccine

	Group		
	1	2	3
No. of rats	20	20	20
Preparation	Antigen/*B. pertussis*	Antigen/*B. pertussis*	Challenge control
Vaccine regime:			
dose in mg worm protein	5	5	–
no. of doses	3	1	–
dose interval in days	7	–	–
days to challenge	10	10	–
Worm burden, GM $\pm$ pooled SE($\log_{10}$):			
day 6	1150 ± 0.03[a]	1303 ± 0.03[a]	1151 ± 0.03[a]
day 10	29 ± 0.18[a]	68 ± 0.18[a]	1143 ± 0.18[b]
% protection	97	94	–
Mast cells/VC, GM $\pm$ pooled SE($\log_{10}$)			
day 6	4 ± 0.13[b]	3 ± 0.13[b]	1 ± 0.13[a]
day 10	8 ± 0.1[c]	3 ± 0.1[b]	1 ± 0.1[a]
Reaginic antibody titre			
day 6	1/1024	1/256	0
day 10	1/4096	1/1024	0

Different subscripts indicate different groups by the studentised range test at a 5% significance level.

Discussion

The results of the present study have shown that significant levels of protection can be obtained in rats against the nematode *N. brasiliensis* using a killed adult worm antigen extract. This confirmed the findings of several other groups of workers (Chandler, 1932; 1936; Watt, 1943; Thorson, 1951; 1953; Denham, 1968; 1969a; Poulain et al., 1976) apart from Ogilvie (1967) who failed to produce any resistance using an adult worm extract of *N. brasiliensis*. One possible explanation for this discrepancy was that the dose of antigen used for immunization in Ogilvie's (1967) experiments was too low.

There are numerous studies with several other nematode parasites where the use of killed parasite antigen or their metabolic products for immunization resulted in significant levels of protection. These include: *Trichinella spiralis* (McCoy, 1935; Campbell, 1955; Chute, 1956; Chipman, 1957; Ewert and Olson, 1960; 1961; Denham, 1967; Despommier and Muller, 1969; Larsh et al., 1970; Vernes, 1976; Despommier et al., 1977), *D. viviparus* (Jarrett et al., 1960b; Wade et al., 1961; Wade et al., 1962; Silverman et al., 1962; Robinson, 1967), *Trichostrongylus colubriformis* (Silverman et al., 1962; Connan, 1965; Denham 1969b; Rothwell and Love, 1974; Rothwell, 1978), *Haemonchus contortus* (Ozerol and Silverman, 1970; Silverman and Paterson, 1960; Scott et al., 1971), *Oesophagostomum radiatum* (Keith and Bremner, 1973), *Ostertagia circumcincta* (Rose 1976; 1978), *A. caninum* (Thorson, 1956), *Nematospiroides dubius* (Van Zandt, 1962; Cypess, 1970), *Trichuris muris* (Wakelin and Selby, 1973; Jenkins, 1976; Jenkins and Wakelin, 1977), *Ascaris suum* (Fallis, 1948; Lejkina, 1953; Soulsby, 1957; 1963; Rhodes et al., 1965; Crandall and Arean, 1965; Guerrero and Silverman, 1969; 1971; Bindseil, 1969; Stromberg and Soulsby, 1977), *Ascaridia* species (Rebrassier and McCrory, 1931; Eisenbrant and Aekert, 1940, both quoted by Ershov, 1959), *Strongyloides papillosus* (Silverman et al., 1962), *Strongyloides ratti* (Sheldon, 1973) and *Litomosoides carinii* (MacDonald and Scott, 1953). These results were obtained in a wide range of laboratory and domestic animal hosts including cattle, sheep, pigs, dogs, mice, rats, rabbits, guinea pigs and chickens.

Significant levels of protection have also been achieved with several cestode parasites using killed worm antigen or their metabolic products in both definitive and in intermediate hosts. Sheep have been immunized against *Cysticercus tenuicollis* (Gemmell, 1969), *C. ovis* (Gemmell, 1969; Rickard and Bell, 1971; Rickard et al., 1976; Rickard et al., 1977) and *Moniezia expansa* (Seddon, 1930; Puklov and Velitchkin, 1936, quoted by Ershov, 1959), while cattle have been successfully protected against *C. bovis* (Gallie and Sewell, 1976; Rickard and Adolph 1976; Rickard et al., 1977). Furthermore, significant levels of protection have been achieved in rats against *C. fasciolaris* (Miller, 1930; 1931a; 1931b; 1932; Campbell, 1936; Kwa and Liew, 1977) and in rabbits against *C. pisiformis* (Miller and Kerr, 1932; Kerr, 1935; Heath, 1976). Turner et al.

Table 8. Studies in which non-living material has been successfully used for immunization

Species	Parasite	Antigen preparation	Adjuvants	% protection	Authors
Guinea pig	T. colubriformis	Soluble extract or metabolic products* of fourth stage larvae	Freund's complete**	>90	Rothwell and Love (1974)
Guinea pig	T. colubriformis	Soluble extract of fourth stage larvae	Aluminium hydroxide	>90	Rothwell (1978)
Guinea pig	D. viviparus	Lyophilized third stage, fourth stage larvae and their metabolic products	Aluminium hydroxide	>90	Silverman et al. (1962)
Guinea pig	A. suum	Killed third stage larvae and their metabolic products	Freund's complete	>90	Soulsby (1963)
Mouse	T. spiralis	Extract of oesophageal gland of the parasite	Freund's complete	>90	Despommier and Muller (1969)
Mouse/Mini pig	T. spiralis	Metabolic products of muscle larvae	Freund's complete** Corynebacterium parvum**	>90	Vernes (1976)
Mouse	T. muris	Extract of adult worms	Freund's incomplete	>90	Wakelin and Selby (1973)
Mouse	T. muris	Extract of adult worms Metabolic products of adult worms	Freund's incomplete Freund's incomplete	>90 >90	Jenkins and Wakelin (1977)
Rabbit	S. papillosus	Lyophilized third stage, fourth stage larvae and their metabolic products	Aluminium hydroxide	>90	Silverman et al. (1962)
Rat	N. brasiliensis	Metabolic products of adult parasites	B. pertussis	>90	Denham (1969a)
Rat	S. ratti	Heat killed larvae	None	>90	Sheldon (1937)
Sheep	C. ovis	Metabolic products of activated embryos	Freund's complete	100	Rickard and Bell (1971)
Sheep	C. ovis	Metabolic products of Taenia ovis larvae	Freund's incomplete	>90 100	Rickard et al. (1976) Rickard et al. (1977)
Sheep	C. tenuicollis	Formalized or frozen activated embryos	None	>90	Gemmell (1969)
Sheep	M. expansa	Ground up proglottids	None	100***	Seddon (1930)
Bovine	C. bovis	Metabolic products of Taenia saginata larvae	Freund's incomplete	>90 to 100	Rickard & Adolph (1976) Rickard et al. (1977)
Bovine	C. bovis	Proglottid homogenate of Taenia saginata	Freund's complete	100	Gallie & Sewell (1976)
Rat	C. fasciolaris	Somatic antigen of strobilocerci. Metabolic product of strobilocerci Purified somatic antigen of strobilocerci (MW = 140,000 daltons)	Freund's complete Freund's complete Freund's complete	>90 >90 100	Kwa and Liew (1977)
Rabbit	C. pisiformis	Killed larvae with metabolic products	None	>90	Heath (1976)
Mouse	H. nana	Adult worm homogenate	Aluminium hydroxide	>90	Coleman et al. (1968)

* Metabolic products are antigens obtained from in vitro cultures of live parasites.
** Adjuvant used with no obvious effect.
*** Only one animal studied.

(1932, 1936) were able to produce high levels of protection in dogs against *Ecchinococcus granulosus.* Using an adult worm preparation, both Larsh (1944) and Coleman et al. (1968) were able to produce significant levels of protection in mice to *Hymenolepis nana,* while Kowalski and Thorson (1972) stimulated protection in mice against *Mesocestoides corti* using metabolic products and a soluble somatic antigen.

Attempts to induce resistance to trematodes by artificial immunization with killed worm antigen have met with very limited success and have often given conflicting results. Adult worm antigen or metabolic products of *Schistosoma japonicum* produced a limited reduction in worms in rabbits (Kawamura, 1929), dogs (Kawamura, 1929; Ozawa, 1930) and in mice (Lin et al., 1954; Sadun and Lin, 1959; Sadun, 1963), whereas, in a study involving 2 monkeys Vogel and Minning (1953) failed to stimulate any protective immunity. With *S. mansoni* limited success has been reported in mice and rats using adult worm preparations (Watts, 1949; Sadun, 1963; Sadun and Bruce, 1964), soluble cercarial immunogen (Phillips et al., 1978) or metabolic products of cercariae and adults (Kagan, 1958; Levine and Kagan, 1960; Murrell and Clay, 1972). Several other groups have failed completely (Thompson, 1954; Ritchie et al., 1962) or obtained inconsistent results (Sher et al., 1974; Murrell et al., 1975).

Published results with other trematodes using killed worm antigen are few and probably reflect the limited success obtained. Attempts to produce resistance to infection with *Fasciola hepatica* using non-living whole fluke preparations or their extracts have given equivocal results. Some workers have reported measurable acquired resistance in rabbits (Kerr and Petkovich, 1935; Shibanai et al., 1956), in mice (Lang, 1976; Lang and Hall, 1977) and in sheep (Ershov, 1959), as judged by reduction in the number of adult worms recovered. Others have failed to substantiate these results (Hughes, 1962). In other studies, some evidence of immunity was obtained in rabbits as judged by reduction in worm size and fecundity but not worm numbers (Urquhart et al., 1954; Healy, 1955; Ross, 1967). Our own preliminary observations in rats have shown that partial but significant levels of protection can be achieved against *F. hepatica* providing large amounts of adult fluke protein and an adjuvant are used.

In the above studies, a wide range of parasite preparations were used to induce protective immunity. These included parasite antigens prepared and inactivated using a variety of methods, including physical and chemical procedures, as well as a range of worm extracts. In addition, there are several reports in which larval or adult worm metabolic products, prepared in vitro, were shown to have protective qualities, in some cases of a very high nature. Furthermore, it was found that at least some of the antigens concerned with protective immunity were likely to have originated from ducted glands in the parasite and were possibly enzymes. Thus, extracts from the anterior or stichosomal region of several nematodes including *A. caninum* (Thorson, 1956), *T. spiralis* (Despommier and Muller, 1969), *O. radiatum* (Keith and Bremmer, 1973) and *T. muris*

(Wakelin and Selby, 1972; Jenkins and Wakelin, 1977) have been shown to stimulate significant levels of protection. The speculation that these protective factors are secretory and might represent enzymes has been supported by the findings of Rhodes et al. (1965) and Edwards and Gutteridge (1968) who were able to stimulate protection using malic dehydrogenase extracted from *A. suum* and *N. brasiliensis* respectively. In a more recent study, however, Rothwell and Merritt (1975) found that the capacity of various extracts of *T. colubriformis* to stimulate protective immunity was not related to their acetylcholinesterase content and that a highly purified fraction of acetylcholinesterase was not effective.

In the majority of studies on immunization with non-living worm material it should be emphasized that the levels of protection achieved, although significant, were in most cases not as high as those obtained with attenuated parasites (Urquhart et al., 1962). However, there are reports in which very high levels of protection have been achieved showing that killed antigen, at least from some parasites, is highly immunogenic and has potential application (Table 8). It is worth noting in these studies that in 13 cases metabolic products were employed in the vaccine, in 16 adjuvant was effectively used, while the dose of antigen was carefully titrated in at least 5 studies.

In the present studies, it was found that several factors influenced protection and if properly manipulated the level of protection achieved by killed adult worm antigen extract was consistently increased to levels of more than 90%. These factors included the use of adjuvant, the quantity of worm protein given, the number of doses employed and the interval between them, and the route of administration of the antigen.

In designing a vaccine regime, the choice of adjuvant is likely to be important and should be based on an understanding of the host's effector mechanisms in dealing with the parasite and also its site of action. *In nippostrongylosis*, for example, there is evidence that worm expulsion is associated with a worm allergen – mast cell – reaginic antibody interaction at the gut level (Barth et al., 1966; Murray, 1972). It has been found in the intestinal mucosa of the rat infected with *N. brasiliensis* that mast cell discharge with the release of vasoactive compounds is associated with the opening up of intercellular pathways between endothelial cells and between epithelial cells, thereby creating enhanced mucosal permeability to macromolecules (Murray et al., 1971). In the intestinal mucosa of the rat most of the IgE is associated with mast cells and not plasma cells (Mayrhofer et al., 1976). Thus, it is possible to envisage a highly specific role for IgE and mast cells at mucous surfaces, namely, that of facilitating translocation of protective antibody or other effector substances across the epithelial sheet of the mucous membrane. This hypothesis has been supported by findings of Steinberg et al. (1975) who showed that IgE facilitated protective antibody release across the vascular barrier and termed it the "gateway" antibody. Thus, because of its well established ability to potentiate reaginic antibody reactions

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(Mota, 1958), *B. pertussis* was selected as the adjuvant for the present investigation. As a result of its use with killed adult worm extract in this study, it was possible to increase protection to levels consistently in excess of 90%.

In previous studies on immunization against helminth infection using non-living parasite material, adjuvants have occasionally been used sometimes with significant effect. Denham (1967), studying *T. spiralis* in the mouse, found that both Freund's complete adjuvant and *B. pertussis* increased protection levels, although *B. pertussis* was superior. Denham (1968, 1969a) went on to show in a preliminary report the effectiveness of *B. pertussis* as an adjuvant in vaccination against *N. brasiliensis* in rats. The use of aluminium hydroxide enhanced protection levels achieved in guinea pigs against *D. viviparus* and in rats against *S. papillosus* (Silverman et al., 1962). Aluminium hydroxide was also effective when used in a vaccine against *T. colubriformis* in guinea pigs (Rothwell, 1978), whereas Freund's complete and incomplete adjuvant had little effect (Rothwell and Love, 1974) indicating that the choice of the correct adjuvant is critical. On the other hand, Wakelin and Selby (1973) found that the use of Freund's incomplete or complete adjuvant significantly increased protection to *T. muris* in mice.

It should also be emphasised that not only is the choice of adjuvant critical but so too is the dose of adjuvant, its route of administration and its time of administration in relation to antigen and dose of antigen; all of these factors are important in deciding which effector arm of the immune response is stimulated (Leskowitz and Waksman, 1960; Dresser and Philips, 1973; WHO, 1976). The number of adjuvants available for use in potential helminth vaccines is limited and it is generally agreed that more should be developed (WHO, 1976). Helminths themselves are able to induce a wide range of responses in the host's immune system, e.g., the nematode *N. brasiliensis* has been shown to potentiate a specific immunoglobulin class response to an heterologous protein antigen (Orr and Blair, 1969). Extending this, Jarrett (1972) used a *N. brasiliensis* infection superimposed on a *F. hepatica* infection in the rat to potentiate the reaginic antibody response to *F. hepatica*. Thus, parasites or their extracts might offer a rich source of potential compounds which could be used as adjuvants.

Another factor shown in the present study to have a critical influence on protection was the dose of antigen employed; there was a direct correlation between the dose of antigen and the level of protection achieved. This finding was in agreement with the results of several other groups including Turner et al. (1936) with *E. granulosus* in the dog, Silverman et al. (1962) with *D. viviparus* in the guinea pig. Despommier et al. (1977) with *T. spiralis* in mice, Poulain et al. (1976) with *N. brasiliensis* in rats, Rothwell and Love (1974) and Rothwell (1978) with *T. colubriformis* in guinea pigs, Lang and Hall (1977) with *F. hepatica* in mice and Kwa and Liew (1977) with *T. taeniaeformis* in rats.

Under most circumstances, it is usually necessary to give 2 or more spaced antigenic stimuli to achieve a good immune response. Furthermore, it has been

found that a suitable time interval between antigenic stimuli and adequate secondary antigen doses is important in induction of an high secondary response (Nakashima et al., 1974). Thus, in the present study on helminth protective immunity it was shown that 2 or more vaccine doses gave significantly better results than one. The time interval between the doses was also confirmed to be critical in that the level of protection achieved when the vaccine was given at monthly intervals was significantly less than when given at weekly intervals. Similarly, Silverman et al. (1962), studying vaccination against *D. viviparus* in guinea pigs, found that the interval between vaccine doses was important and in their studies established that 21 days was the optimal period.

In developing a vaccine strategy, it is essential to consider the site inhabited by the parasite in order that the immune response can be maximised at that particular location. Several factors are known to influence the site at which the immune response is operative; these include route of administration, dose of antigen and whether the antigen is live or dead (Spencer et al., 1974; Bienenstock, 1975; Frederick and Bohl, 1976). In the present study, the level of protection was found to be influenced by the route of immunization. The intraperitoneal route gave the best results and was superior to the subcutaneous and the oral route which was poorest. Vernes (1976), studying vaccination against *T. spiralis* in mice and minipigs using metabolic antigens from muscle larvae, confirmed that the route of vaccination was important but that the oral route gave superior results to the subcutaneous route. On the other hand, Rothwell (1978) found the oral route for administration of vaccine to be inferior to subcutaneous, intradermal, intraperitoneal and intraduodenal routes. The level of protection obtained in the present experiment using the oral route was disappointing when one considers that adult *Nippostrongylus* parasites inhabit the small intestine and that the antigen used was apparently only effective against adult parasites. The results probably reflect degradation of worm protein by the gastrointestinal tract and possibly the potential difficulty in establishing good immunological memory at local mucous surfaces (Murray, 1973; Waldmann and Ganguly, 1975). To overcome this problem it might be necessary to administer antigen repeatedly at the local level in order to reach and maintain satisfactory levels of immunity. Alternatively, it is known that the administration of large amounts of antigen parenterally will stimulate both systemic and local responses (Spencer et al., 1974; Bienenstock, 1975) and it would appear that this was what was achieved by the use of the intraperitoneal route in the present study.

It is usually considered that one of the potential disadvantages of the use of a killed antigen, is a rapid waning of immunity. However, in the present study, it was found that the use of a triple dose of worm antigen with adjuvant given intraperitoneally stimulated high levels of immunity demonstrable for at least 2 months after the last vaccine inoculation. Despommier and Wostmann (1968) were able to demonstrate persistence of protective immunity in mice by in-

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traperitoneal implantation of diffusion chambers containing *T. spiralis* larvae; strong immunity still existed 6 months after removal of the chambers. In one of the first demonstrations of artificially-stimulated protective immunity, Miller (1932) found that good protective immunity lasted in the rat for as long as 167 days after the last immunizing dose with a dried powdered adult worm preparation of *T. taeniaeformis*, while, using culture antigen of *T. ovis* larvae in Freund's incomplete adjuvant to vaccinate sheep, Rickard et al. (1977) showed that high levels of protective immunity lasted for at least one year after vaccination.

The use of antigen prepared from adult *Nippostrongylus* worms produced no apparent cross reactivity to the larval challenge in that in immunized rats the expected adult population became established in the small intestine, as judged by the challenge controls. Only then did the immunological effector mechanisms potentiated by vaccination become operative. It is significant to observe on the basis of this finding that the adjuvant *B. pertussis* had helped to potentiate effector mechanisms operative at the level of the mucous surface. Thus, the site of action of protective effector mechanisms induced by this immunization regime is distinct from that which exists in rats subjected to a reinfection regime where it has been shown that increased resistance is due, at least in part, to the fact that a significant proportion of the challenge infection is killed as larvae in the lungs (Jarrett et al., 1968).

While in certain cases considerable success has attended the use of irradiated parasitic vaccines, there are a large number of helminth infections in which the use of irradiated parasites has produced significant but not commercially exploitable levels of protective immunity (Urquhart et al., 1962). It is possible that, if the parameters highlighted in this paper as being important in protective immunity were considered, this situation might be improved.

The potential advantages and disadvantages involved in the use of a dead or living attenuated vaccine are well recognized. Dead vaccines are likely to have a good shelf life and less likely to produce any pathological side effects, both disadvantages of attenuated vaccines. On the other hand, attenuated vaccines are likely to stimulate stronger and longer lasting immunity. Perhaps the use of dead or living immunogens should not be considered mutually exclusive and their possible use in combination be examined, e.g., a priming inoculation of killed antigen followed by a booster with attenuated antigen might stimulate a strong and long lasting immunity with reduced danger of pathological side effects.

In conclusion, in planning the strategy of any vaccination regime, a basic understanding is required of the host's immune effector mechanisms, as is an appreciation of the logistics and parameters of the immunological engineering programme which might be required. These parameters should include, the nature of the antigen (live or dead), dose of antigen, number of doses and interval between doses of antigen, and the use and choice of adjuvants. The fact that protection, sometimes of a high order, can be achieved using non-living parasit-

ic material should encourage research into the isolation and characterization of helminth protective antigens, the nature of which remains largely unknown at present. It would appear from the evidence gleaned from the literature and described in the present study that metabolic products obtained from in vitro parasite cultures might be a rewarding starting point. The presentation to the host of purified protective antigens rather than the plethora of antigens usually given in most killed antigen preparations, is likely to produce a much more effective protective response.

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