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The Cellular Transfer of Immunity to *Trichostrongylus colubriformis* in an Isogenic Strain of Guinea-Pig

II. THE RELATIVE SUSCEPTIBILITY OF THE LARVAL AND ADULT STAGES OF THE PARASITE TO IMMUNOLOGICAL ATTACK

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Summary. Cells obtained from mesenteric lymph nodes of highly inbred guinea-pigs (Heston strain) resistant to *Trichostrongylus colubriformis* were injected into virgin animals of the same genotype. The adoptively immunized recipients were challenged with 1000 *T. colubriformis* larvae 4 days after transfer and slaughtered at intervals which correspond to critical times in the development of the parasite. Differential worm counts carried out on specimens of intestine showed that a sharp decline in the number of parasites occurred between days 7 and 9. This period corresponds to the time required for the parasite to develop to the fourth larval stage.

Variation of the time interval between cell transfer and challenge showed that immune cells transferred on the day of challenge and on days 4, 6 and 8 after challenge inhibited the development of infection to patency, while cells injected on day 10 were without effect. This observation confirmed that the fourth larval stage of the parasite is uniquely susceptible to the immunological attack initiated by the transferred cells and showed that these cells are effective within 24–48 hours after injection. This latter finding excludes the possibility of active participation in the response by the recipient.

Resistance can be transferred by spleen cells and by cells obtained from lymph nodes other than the mesenteric nodes which drain the site of infection. However the local nodes are more effective and resistance was regularly transferred with as few as 10×10^6 cells injected intravenously.

INTRODUCTION

Although the transfer of cellular components of the immune response has been used as a procedure to analyse the mechanism of first, the homograft response to both tumour cells (Mitchison, 1955) and orthotopic skin grafts (Billingham, Brent and Medawar, 1954) and secondly, delayed hypersensitivity (for review, see Lawrence, 1956), few reports have been made of attempts to transfer resistance to actual infection using this technique. This procedure is not necessary for bacterial and viral infections since such organisms are usually affected by the appropriate humoral antibody and the resistance status of the

animal is fairly accurately indicated by the level of serum antibody. It is now generally accepted that such humoral factors are not involved in the homograft response, at least for the destruction of solid tissues such as skin (Brent, Brown and Medawar, 1959), and that the level of serum antibodies does not reflect the immunological status of the animal. This latter finding is also frequently encountered by investigators in the field of immunity to metazoan parasites and particularly to helminths. The similarity between helminth immunity and the homograft reaction has recently been strengthened by the finding that resistance to *Trichostrongylus colubriformis* in an isogenic strain of guinea-pig could be transferred to 'virgin' animals with local mesenteric lymph node cells, whereas injection of up to 10 ml of immune serum into a single recipient was not effective (Wagland and Dineen, 1965). The only other report of the cellular transfer of resistance to a parasite which has come to our notice is that of Larsh, Goulson and Weathery (1964) who showed that peritoneal exudate cells, obtained from a donor mouse infected with *Trichinella spiralis*, caused the accelerated removal of adult worms when injected into a recipient. In addition, Long and Pierce (1963) have suggested that cellular rather than humoral factors may also be involved in acquired immunity to *Eimeria tenella* in fowls. They based this conclusion on the finding that birds in which the development of the bursa of Fabricius was suppressed by hormonal treatment *in ovo*, failed to develop serum antibodies, but nevertheless were immunized successfully.

In the studies described in the present communication, we have investigated the competence of lymphoid cells derived from different tissues in addition to the mesenteric lymph nodes which drain the site of infection to transfer immunity to *T. colubriformis* in the guinea-pig. The relative effectiveness of the mesenteric node cells and other lymphoid cells in this respect has also been studied. In addition, by varying the time between cell transfer and challenge, information has been obtained concerning the susceptibility of the several stages of development of the helminth to immunological attack.

MATERIALS AND METHODS

Experimental animals

Both male and female guinea-pigs 3–4 months of age, were used in the experiments and, except where indicated, were the progeny of highly inbred parents (Heston strain). The stock colony has been regularly tested for histocompatibility between members by reciprocal transplantation of orthotopic skin grafts.

Infective larvae

The infective larvae used to immunize the donor guinea-pigs and for challenging the recipient guinea-pigs after cell transfer were obtained from cultures prepared from the faeces of a sheep infected with *T. colubriformis*. The faecal cultures were incubated at 27° for 7 days before washing off the infective larvae which migrated out of the cultures. A total count was made of the infective larvae using a dilution technique. Appropriate aliquots, each calculated to contain 1000 larvae, were pipetted into tubes and stored at 4°. Prior to dosing the guinea-pigs, the larvae were allowed to stand at room temperature for 2 hours and any tubes containing larvae which were not vigorously motile were discarded.

Administration of larvae and faecal worm egg counts

These procedures were carried out in accordance with the description given by Wagland and Dineen (1965).

Preparation of the transfer cells

The donor guinea-pigs received a course of a minimum of four infections each with 1000 larvae given over a 4 week period and were slaughtered 7 days after the last dose. The peritoneal cavity was exposed and the donor lymphoid tissues removed with aseptic precautions and placed into Hanks's solution. The excised tissue were finely diced with scissors and single cells and small clumps of cells released by gentle pipetting. The single cells were separated from the small tissue fragments by differential sedimentation. The supernatant containing mostly single cells was then run through a very fine stainless steel sieve and the material that passed through the sieve was concentrated by centrifugation at 500 rev/min for 10 minutes. The concentrated single cells were resuspended in a known volume of Hanks's solution and a total cell count made on a convenient dilution in a haemocytometer. The volume of the single-cell suspension was adjusted so that the desired number of cells were contained in 1 ml for injection. The single-cell suspension was injected into one of the blood vessels in the ear of the recipient guinea-pigs using a 1-ml tuberculin syringe and 27 gauge needle. The animals were lightly anaesthetized with ether and the ears transilluminated to facilitate intravenous injection. Except where indicated, the recipient guinea-pig and a paired untreated control animal were each infected with 1000 *T. colubriformis* larvae 4 days after injection of the lymphoid cells. Faecal samples were obtained from individual guinea-pigs by housing each animal separately in cages designed so that the faecal pellets would pass easily through the wires of the holding cage. For most of the experiments faecal egg counts were used to assess the effect of the immunological response produced by the transferred cells. Thus, faecal worm egg counts were estimated regularly after the prepatent period (day 15) until termination of infection with *T. colubriformis* which normally occurs in the guinea-pig at about day 30.

Worm counts

Worm counts were made by slaughtering the guinea-pig and isolating the gastrointestinal tract into three sections, i.e. (1) stomach; (2) small intestines, including duodenum and ileum; and (3) large intestine and caecum. The contents of each section were washed into separate bottles and the intestines opened and incubated for 1 hour in 1 per cent hydrochloric acid to dislodge the immature worms from the mucosa. The two fractions were combined and formalinized. After fixation the contents of each bottle were poured onto a 200 mesh sieve to allow small particles and coloured fluid to pass through. The material retained by the sieve was washed off and examined for the presence of worms. Usually all the material was examined and the counts for the three sections of the alimentary tract of each guinea-pig combined to give the total worm count. The worms were classified as either fourth stage or adult.

It was necessary to examine the whole intestine as the results of previous workers showed that *T. colubriformis* is often found in parts of the intestine other than the small intestine (Gordon, Mulligan and Reinecke, 1960).

EXPERIMENTAL RESULTS

THE DEVELOPMENT OF CHALLENGE INFECTIONS IN ADOPTIVELY IMMUNIZED GUINEA-PIGS

The mesenteric lymph nodes of sixteen actively immunized guinea-pigs were removed and finely diced with scissors. The small tissue fragments and single cells were concentrated by light centrifugation and resuspended in 5–10 ml of Hanks's solution. All the lymphoid

tissue from one donor was injected intraperitoneally into one recipient guinea-pig. The sixteen injected animals and sixteen untreated control animals were challenged with 1000 *T. colubriformis* 4 days after the recipients were injected with cells. The recipients and their paired controls were slaughtered at intervals after challenge. Thus, three pairs were slaughtered on days 4, 7, 9 and 14 and the remaining four pairs slaughtered between days 17 and 22. These time intervals were selected because they correspond to moulting periods in the development of *T. colubriformis*. Although no critical studies have been made on the development rate of *T. colubriformis* in the guinea-pig, it is known that the prepatent period is similar in guinea-pigs and the natural hosts. The data obtained by Monnig (1927) for infection in lambs, and Douvres (1957) for infection in cattle, indicate that transition from the second to the third parasitic stage takes place within 24–48 hours after ingestion of infective larvae, and ecdysis to the fourth stage occurs between days 4 and 5. The final ecdysis to the adult stage occurs between days 8 and 10 after infection. Thus, the slaughter periods were chosen in the present experiments to show which stage was most susceptible to the immune response.

The total number of worms recovered from the adoptively immunized and the control guinea-pigs slaughtered on days 4, 7, 9 and 14 and subsequent to day 17 are shown in Table 1.

TABLE 1

WORM BURDENS OF ADOPTIVELY IMMUNIZED GUINEA-PIGS AFTER CHALLENGE WITH 1000 *Trichostrongylus colubriformis* LARVAE

Days after challenge when slaughtered	Total number of worms recovered in:	
	Recipient guinea-pigs	Control guinea-pigs
4	152	381
4	216	259
4	326	249
	Mean 231	*Mean* 296
7	415	257
7	90	293
7	300	353
	Mean 268	*Mean* 301
9	107	291
9	15	322
9	171	389
	Mean 97	*Mean* 334
14	19	88
14	0	176
14	64	198
	Mean 28	*Mean* 154
17	0	149
20	0	101
21	12	80
22	0	104
	Mean 3	*Mean* 108

The total number of worms recovered from guinea-pigs injected with mesenteric lymphoid tissue and challenged 4 days later with 1000 *T. colubriformis* larvae.

This table shows that there was little difference between the total worm burden of the recipient and control guinea-pigs slaughtered on days 4 and 7 after challenge. No reduction in worm burden in either the treated or control guinea-pigs occurred within this period.

It is evident from these results that the challenge infections developed to the fourth stage in both control and adoptively immunized animals. However, the worm burden was greatly reduced in the treated guinea-pigs slaughtered 9 days after challenge. A mean of ninety-seven worms was found in the recipient animals on this day compared with a mean of 334 in the controls. This marked reduction in worm numbers in the recipient guinea-pigs between day 7 (268 worms) and day 9 (ninety-seven worms) shows that elimination or rejection of part of the infection occurred during this period. Almost all the worms found in the control guinea-pigs were recovered from the small intestine whereas one-third of the worms recovered from the recipient guinea-pigs were found in the large intestine or caecum.

A marked difference in total worm count between the recipient and control guinea-pigs was again apparent in the animals slaughtered on day 14. A mean of 154 worms was recovered from the controls whereas only a mean of twenty-eight worms was recovered from the recipient guinea-pigs. The distribution of the worms between the small and large intestine was similar in both groups of guinea-pigs. Worms were not found in three of the four treated guinea-pigs slaughtered between days 17 and 22 whereas a mean count of 108 worms was found in the control guinea-pigs.

THE EFFECTIVENESS OF MESENTERIC LYMPHOID CELLS INJECTED AT INTERVALS AFTER INFECTION OF THE RECIPIENT GUINEA-PIGS

In the previous experiments it was found that the late fourth stage worms were eliminated from guinea-pigs infected 4 days after adoptive immunization with mesenteric lymphoid cells. Thus, a marked difference was found in the worm counts of the injected guinea-pigs and the control guinea-pigs slaughtered 9 days after infection. These results indicate that either an extended latent period of about 11 days was necessary before the immune response became effective, or that the late fourth stage worms were more susceptible than the earlier developmental stages. The present experiment was carried out to obtain further information on the time relationship between injection of the lymphoid cells and the susceptibility of the various stages of development of the parasite.

A total of forty-eight guinea-pigs were each given 1000 *T. colubriformis* larvae on day 0 and divided into six groups each containing eight animals. On the same day, four of the guinea-pigs in the first group were injected intravenously with 100×10^6 mesenteric lymphoid cells. Four animals in each of the remaining groups were injected with the same number of cells from freshly slaughtered immune donor guinea-pigs at intervals of 4, 6, 8, 10 and 12 days after initial infection. The twenty-four guinea-pigs not injected with cells were used as controls for comparison with the recipient animals so that the subsequent faecal worm egg counts would show on which days the transferred cells were effective in inhibiting development of the parasite. Faecal worm egg counts of the individual animals were estimated on samples obtained at regular intervals after day 15 until day 30.

The relative fecundity of the infections in each of the injected and control guinea-pigs was estimated by measuring the area subtended by the faecal worm egg counts between days 17 and 27 after infection. These values are shown in Table 2.

This table shows that the infections were greatly reduced in the guinea-pigs injected with cells on the same day as the larvae were given, and at 4, 6 and 8 days after infection. The mean fecundity of infections in these guinea-pigs ranged between 12 and 51 whereas the controls had values of between 235 and 379. However, the fecundity of infections in the guinea-pigs injected with cells on days 10 and 12 were similar to the control animals.

TABLE 2

THE EFFECT OF TRANSFERRING MESENTERIC LYMPHOID CELLS AT INTERVALS AFTER INFECTION OF RECIPIENT GUINEA-PIGS

Cells injected on day number	Relative fecundity of infection in:*	
	Recipient guinea-pigs	Control guinea-pigs
0	12	934
	0	357
	37	226
	0	0
	Mean 12	*Mean* 379
4	91	278
	65	432
	0	14
	0	259
	Mean 39	*Mean* 328
6	64	264
	52	199
	56	438
	7	38
	Mean 45	*Mean* 235
8	8	230
	0	450
	65	301
	130	332
	Mean 51	*Mean* 328
10	0	19
	310	34
	122	695
	310	255
	Mean 185	*Mean* 251
12	325	78
	193	918
	586	258
	703	309
	Mean 452	*Mean* 391

* The relative fecundity of infections in guinea-pigs injected with 100×10^6 cells days 0, 4, 6, 8, 10 and 12 after challenge. The transferred cells were obtained from the mesenteric lymph node of immune guinea-pigs and were given as a single-cell suspension to the recipient guinea-pigs by the intravenous route. All recipient and control guinea-pigs challenged with 1000 *T. colubriformis* larvae on day 0.

This result shows that the immune response mediated by the transferred cells was not effective against either the early fifth stage or adult worms.

THE NUMBER OF MESENTERIC LYMPHOID CELLS REQUIRED FOR THE CELLULAR TRANSFER OF RESISTANCE TO *Trichostrongylus colubriformis*

An experiment was carried out to determine the minimum number of mesenteric lymphoid cells obtained from an immune guinea-pig, that are required for the effective transfer of resistance. Single cell suspensions were prepared and the volume of the suspension adjusted to give the desired number of cells in 1 ml for intravenous injection. The recipients and their paired control animals were challenged 4 days after injection of the cells. The effect of adoptive immunization was again assessed by comparing the fecundities of infection in the recipient animals at the various dose levels, with the infections that developed in the control animals. The results are shown in Table 3.

TABLE 3

THE EFFECT OF TRANSFERRING GRADED DOSES OF MESENTERIC LYMPH NODE CELLS BY THE INTRAVENOUS ROUTE

No. of cells injected ($\times 10^6$)	Relative fecundity of infection in:	
	Recipient guinea-pigs	Control guinea-pigs
450	0	345
300	0	578
300	0	218
200	0	172
200	0	163
100	109	224
100	205	745
30	155	636
30	0	364
10	0	239
10	24	466
10	0	146
10	25	124

This table shows that the relative fecundities of infections in the recipients, injected with between 10×10^6 and 450×10^6 cells, were much reduced by comparison with the controls. Eggs were not found in the faecal samples examined from the animals injected with 200×10^6 or more cells, but variable results were obtained for the guinea-pigs injected with 100×10^6 cells or less. Four animals which received 10×10^6 cells had very low faecal egg counts (mean fecundity, 12), but three guinea-pigs given 100×10^6 and 30×10^6 cells developed moderate counts after challenge (mean fecundity, 117). All the control animals had moderate to high faecal worm egg counts (mean fecundity, 368).

THE TRANSFER OF RESISTANCE BY CELLS OBTAINED FROM SPLEEN AND DISTAL NODES

The ability of cells prepared from spleen and lymph nodes other than the mesenteric node to transfer resistance was investigated. Cells were obtained from the thoracic, cervical, axillary, inguinal and lumbar lymph nodes. Preparation of single-cell suspensions from these pooled nodes and the spleen was similar to that outlined for preparing the cells from the mesenteric lymph node. The relative fecundity of infections with 1000 *T. colubriformis* larvae given 4 days after the intravenous injection of the cells into the recipient guinea-pigs and the paired untreated controls was determined from the resulting faecal worm egg counts. These values together with the number of cells injected are shown in Table 4.

The relative fecundity of infections in the five guinea-pigs injected with between 130×10^6 and 250×10^6 cells obtained from the spleen of immune donors was reduced in comparison with the values of the control guinea-pigs. The mean relative fecundity of infections in the recipient guinea-pigs was 41 and in the control animals 763.

The relative fecundity of infections in the guinea-pigs injected with between 90×10^6 and 100×10^6 lymphoid cells obtained from nodes distal to the usual site of infection of *T. colubriformis*, was lower in six of the eight recipients than in the control guinea-pigs. These results show that both spleen cells and lymphoid cells obtained from nodes other than the mesenteric are able to transfer resistance.

TABLE 4

THE CELLULAR TRANSFER OF RESISTANCE WITH SPLEEN CELLS AND WITH LYMPHOID CELLS OBTAINED FROM NODES OTHER THAN MESENTERIC

Origin of cells	No. of cells injected ($\times 10^6$)	Relative fecundity of infection in: Recipient guinea-pigs	Control guinea-pigs
Spleen	250	48	D†
	250	60	330
	250	16	751
	160	0	1142
	130	81	829
		Mean 41	*Mean* 763
Lymph nodes other than mesenteric*	100	194	258
	90	18	713
	90	0	503
	90	59	468
	100	79	371
	100	118	871
	100	526	97
	100	47	795
		Mean 130	*Mean* 510

* The lymphoid cells were obtained from nodes other than the mesenteric lymph node and included cells from the thoracic, cervical, axillary, inguinal and lumbar lymph nodes. The transferred cells were given as a suspension of single cells to the recipient guinea-pig by the intravenous route. The relative fecundity of infections given to both the injected and their paired controls 4 days after injection of the cells into the recipient guinea-pigs is shown above.

† Guinea-pig died before infection reached patency.

DISCUSSION

The elucidation of the mechanism of resistance to helminth parasites provides a challenging problem for fundamental immunological study. Attempts to obtain a simple solution of this problem by transferring even massive volumes of serum from resistant to susceptible animals have usually been unsuccessful, although antibodies can readily be demonstrated in these sera. It is necessary, therefore, to explore the possibility that purely cellular components of the immune response may be involved in resistance to helminths and *T. colubriformis* infection in the guinea-pig provides a suitable laboratory system for experimental studies. Several authors (Herlich, Douvres and Isenstein 1956; Herlich, 1958, 1963; Gordon *et al.*, 1960; Poynter and Silverman, 1962) have used the guinea-pig as host for *T. colubriformis*, which is a natural parasite of sheep and cattle. These studies have been hampered by the finding that establishment of infection in outbred guinea-pigs is extremely variable. Fortunately, the highly inbred Heston strain guinea-pig behaves predictably in this respect and one of us (B.M.W.) has maintained cultures of the parasite through twenty-seven generations by serial passage in the Heston strain. However, the primary reason for using isogenic animals in the current experiments is that intervention of the homograft reaction is thereby avoided during attempts to transfer resistance by transplantation of cellular components of the immune response.

In a previous experiment, resistance to *T. colubriformis* was transferred by mesenteric lymph node cells though not by serum obtained from resistant animals (Wagland and Dineen, 1965). In addition resistance was not transferred by the intravenous injection of

100×10^6 mesenteric lymph node cells obtained from non-immune Heston strain guinea-pigs. In the present experiments mesenteric lymph node cells from resistant guinea-pigs were injected into susceptible animals 4 days before challenging them and control untreated guinea-pigs each with 1000 *T. colubriformis* larvae. The recipients and the control animals were slaughtered at intervals after challenge and differential worm counts performed to determine whether the immunological attack was directed against all or a particular stage of the developing parasites. The number of worms found in the recipients of active lymphoid cells and the control guinea-pigs was similar until day 7 to day 9 after challenge, when the counts recorded for the recipient animals declined sharply (see Table 1). This finding is in agreement with that described by Herlich (1963) who observed that a difference occurred between the number of parasites found in immune and non-immune control guinea-pigs at post mortem 5–12 days following challenge, although no such difference was recorded before day 5. As the 7–9-day period coincides with the development of the parasite from the fourth larval stage to the immature adult, it was highly suggestive that the fourth stage was uniquely susceptible to the immunological response mediated by the transferred lymphoid cells. The alternative explanation, that it required 11 days (4 days plus 7 days) for the injected cells to become effective, could not be excluded, although it would be expected that the cells which were derived from a fully resistant animal should be capable of initiating a vigorous immunological attack within a much shorter period after transfer. This conclusion has been confirmed subsequently, and we have shown that it is the fourth larval stage which is most susceptible to attack by the transferred cells. Thus 100×10^6 mesenteric lymph node cells obtained from resistant guinea-pigs were injected into recipient animals on the day of challenge and at 4, 6, 8, 10 and 12 days after challenge. For comparison appropriate untreated control animals were challenged at the same time as the recipient animals so that the faecal worm egg counts of both groups of animals would reveal on which days the transferred cells were effective in suppressing the development of the infections to patency. The results clearly showed that cells injected on the day of challenge and at 4, 6 and 8 days were effective, but cells injected on days 10 and 12, when the challenge infections were expected to have developed to the adult stage, were not (see Table 2). As the cells injected as late as 8 days following challenge infection were effective, it is clear that: (1) It is the fourth larval stage which is susceptible to immunological attack; and (2) the injected cells are effective immediately, or, at least, within 24–48 hours after injection. This finding would seem to exclude active participation in the reaction by the immune response of the recipient (e.g. active immunization by antigens transferred with the cells).

The finding that the fourth larval stage of *T. colubriformis* is susceptible to immunological attack developed by the adoptively transferred lymphoid cells is of some relevance to helminth infection in the natural host. Retardation or arrested development of the fourth larval stage of *Ostertagia* spp. in resistant sheep has been reported by Dunsmore (1961) and in cattle by Michel (1963). Donald, Dineen, Turner and Wagland (1964) have observed a similar phenomenon with *Nematodirus spathiger* in sheep and with *Haemonchus contortus* in sheep (Dineen, Donald, Wagland and Offner, 1966). Thus, immunological attack upon the fourth larval stage of helminth parasites is an important and general manifestation of resistance in the natural host. We wish to emphasize, however, that the unique susceptibility of the fourth stage of the parasite to an immunological response does not necessarily imply that this stage is solely or even most immunogenic.

While resistance can be transferred adoptively with cells obtained from both spleen and

regional lymph nodes other than those draining the site of infection (see Table 4), the mesenteric lymph node cells are highly effective, and resistance to *T. colubriformis* is regularly transferred by the intravenous route with 10×10^6 cells (see Table 3). Further studies (not included in the present communication) have shown that resistance can be transferred with as few as 1×10^6 mesenteric lymph node cells although less regularly. Thus, the sensitivity of the parasite to action initiated by the transferred immune cells compares favourably with the transfer of adoptive immunity in the skin homograft system described by Billingham *et al.* (1954) in which it was found necessary to inject into a recipient all the cells obtained from four regional nodes to obtain reproducible results. Brent, Brown and Medawar (1958) described a 'hypersensitivity reaction' of the delayed type in the skin of recipient guinea-pigs following injection of lymphoid cells obtained from donors sensitized by skin homografts of the same genotype as the recipients. To evoke this response 5×10^6 to 10×10^6 cells were injected intradermally. This reaction may be closely allied to the response elicited in the infected host following adoptive transfer, with the parasite as the antigenic target, rather than allogeneic tissue.

It is not profitable with the information currently available to interpret the phenomenon described in the present communication in precise mechanistic terms. We may recognize, however, that the transferred cells may act either directly in some undefined way upon the parasite, or indirectly, as a result of a response of the organism to an unfavourable environment which inhibits further development. An unfavourable environment may be produced by a hypersensitivity reaction at the site of infection.

The finding that the transferred cells failed to affect the adult worm requires further comment. Elimination of the adult population, which occurs usually at about day 30, may be due to some general physiological component of the environment in the abnormal host. This would not appear to be likely since no such restriction is placed upon development of the parasite which proceeds to patency in the guinea-pig in the same period (15–17 days) as in the sheep and the eggs are fertile. The alternative view is that either many more cells are required to affect the adult than the fourth stage, or, that the component of the immune response, which causes elimination of the mature burden, was not transferred by the procedure used in the current experiments.

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