STUDIES ON ACQUIRED IMMUNITY TO THE DOG HOOKWORM, ANCYLOSTOMA CANINUM.¹

BY

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The studies of Sandground (1928) on Strongyloides stercoralis in dogs, Stoll (1929) on Haemonchus contortus in sheep, McCoy (1931, b) on Trichinella spiralis in rats, Sarles (1932) on Trichostrongylus colubratus in rabbits, Graham, Ackert, and Jones (1932) on Ascaridia lineata in chickens, Winfield (1933) on Heterakis spumosa in rats, and Africa (1931), Chandler (1932), Graham (1934), and Porter (1935, a) on Nippostrongylus muris in rats have all shown that a definite acquired resistance can be established against these forms. Herrick (1928), McCoy (1932, a), and Foster (1935) have reported that a resistance against the dog hookworm, Ancylostoma caninum, was developed from previous infections. Foster believed that the resistance was due to a physiological compensation. It has also been suggested by Darling and by Cort (see Cort, 1932) that the same phenomenon protected the human host in most cases from the acquisition of overwhelming hookworm burdens.

The resistance to all the nematodes mentioned above is expressed in one or more of the following four ways: (1) reduction in the worm burden, (2) delayed development of the worms, (3) reduced size of the worms developing, and (4) lower fecundity. The manner in which the defense mechanism of the host affects the worms so that the resistance is expressed in these ways is not known. All of these nematode parasites except Heterakis spumosa and Haemonchus contortus show a phase during the earlier part of their life cycles when there is intimate contact with host tissue. This phase of the life cycle

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is particularly important in the development of an acquired resistance; therefore it should be investigated separately from the intestinal phase when there is no intimate contact with host tissue.

One method of attacking the problem of acquired immunity to hookworm is the use of an abnormal host. In this type of host the first phase of the life cycle follows the normal course; i.e., after the infective larvae penetrate into the host, they pass by way of the bloodstream to the heart and then to the lungs. Following this the larvae show no further development since they are not able to establish themselves in the intestine. This is an advantage, for it limits the effect of the parasite on the host to that phase of the life cycle in which the most intimate contact with host tissue occurs; thus any resistance produced may be attributed to the intimate contact. A further advantage in the use of abnormal hosts is derived from the ease with which numbers of animals, such as mice, can be handled. On the other hand, it must be recognized that certain limitations are involved in the use of this type of host. The most important of these is that the parasite is not completely adapted to the host. Considering this, the interpretation of the results derived from such experiments must be applied with caution to the normal host-parasite relationships.

The results which will be presented in this paper demonstrate that a true acquired immunity can be established against hookworm and test the suggestion that the intimate association between host tissue and the parasite is important in the production of immunity. This has been done with the dog hookworm, *Ancylostoma caninum*. A true acquired immunity to a lethal dose is demonstrated through repeated infection in an abnormal host, the mouse, where no development to the adult stage occurs. The importance of the tissue contact between host and parasite is demonstrated by comparing the cellular response to a lethal test infection in resistant and non-resistant mice.

**Materials and methods.**

Ordinary laboratory white mice were used throughout these experiments. They were either bred in the laboratory or purchased as young adults so that none of them were over 6 months old when placed on experiment.

The strain of the dog hookworm, *Ancylostoma caninum*, used in these experiments is one that has been maintained in this laboratory for approximately 10 years. Every care was taken to ensure that the larvae to be used in infecting the mice were strong and healthy.
Fresh cultures were used whenever possible and the larvae were always reisolated in a miniature Baermann apparatus before they were used for infection purposes, in order to eliminate the old and weakly moving larvae. In all infections where less than 250 larvae were used, they were counted individually; otherwise the larvae were counted by the dilution method as described by Scott (1928).

When the mice were to be infected by skin they were first anesthetized with “avertin.” Then the abdominal hair was clipped short and the larvae, in a small amount of water, were applied to the area. The mouse was kept under anesthesia for at least one-half hour and the area upon which the larvae were applied was kept moist during that time. In mice that were repeatedly infected by skin, no attempt was made to utilize a different area for each infection.

Mice that were infected by mouth were lightly anesthetized with ether and a catheter, such as described by Winfield (1933), inserted, the larvae thus being injected in a small amount of water into the lower part of the esophagus.

The tissues removed from the mice, for study of the cellular response to infection, were prepared according to a standard histological procedure for making paraffin sections. They were fixed in Bouin’s fluid, dehydrated in alcohol, cleared in xylol and imbedded in paraffin. Serial sections 10 micra in thickness were stained with hematoxylin and eosin. A few sections were treated with Wright’s blood stain.

RESULTS.

Experimental demonstration of acquired immunity in mice to A. caninum.

Average lethal dose. One of two methods can be used to determine whether any resistance is produced through previous infections. The first of these methods requires the killing of the host at definite intervals after infection and comparing the number of larvae recovered from the lungs of repeatedly infected and previously uninfected animals. Wagner (1933) has used this method in determining resistance to ascaris in mice. The other method is the use of an average lethal dose. Since the first method seemed to be not only tedious but also subject to considerable experimental error, it was decided to set up an approximately average lethal dose. The term average lethal dose is substituted for the term minimum lethal dose since Topley (1933) has pointed out that the latter is a quantity which cannot be measured and suggests the substitution of the former.
TABLE 1.

Data showing the number of larvae of the dog hookworm, Ancylostoma caninum, which is an approximate average lethal dose for mice.

<table>
<thead>
<tr>
<th>Skin infection</th>
<th>Mouth infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse number</td>
<td>Number of larvae given</td>
</tr>
<tr>
<td>1</td>
<td>6,000</td>
</tr>
<tr>
<td>2</td>
<td>10,000</td>
</tr>
<tr>
<td>3</td>
<td>20,000</td>
</tr>
<tr>
<td>4</td>
<td>1,000</td>
</tr>
<tr>
<td>5</td>
<td>2,100</td>
</tr>
<tr>
<td>6</td>
<td>1,000</td>
</tr>
<tr>
<td>18</td>
<td>3,000</td>
</tr>
<tr>
<td>19</td>
<td>4,000</td>
</tr>
<tr>
<td>20</td>
<td>5,000</td>
</tr>
<tr>
<td>22</td>
<td>3,000</td>
</tr>
<tr>
<td>23</td>
<td>3,000</td>
</tr>
<tr>
<td>24</td>
<td>2,550</td>
</tr>
<tr>
<td>25</td>
<td>2,550</td>
</tr>
<tr>
<td>26</td>
<td>2,550</td>
</tr>
<tr>
<td>38</td>
<td>3,200</td>
</tr>
<tr>
<td>39</td>
<td>3,200</td>
</tr>
<tr>
<td>40</td>
<td>3,000</td>
</tr>
<tr>
<td>49</td>
<td>2,900</td>
</tr>
<tr>
<td>50</td>
<td>3,000</td>
</tr>
<tr>
<td>57</td>
<td>4,500</td>
</tr>
<tr>
<td>107</td>
<td>2,000</td>
</tr>
<tr>
<td>108</td>
<td>2,000</td>
</tr>
</tbody>
</table>

Table 1 shows the results of attempts to determine the average lethal dose by the infection of twenty-two mice by skin with 1,000 to 20,000 larvae of ten mice by mouth with 1,000 to 7,750 larvae. In both series of infections all of the mice died when 3,000 or more larvae were administered, death usually occurring within 7 days. Autopsy of these mice revealed that they died from severe hemorrhage into the lung alveoli, probably due to the larvae breaking through the capillary bed of that organ and thus producing verminous pneumonia. Further analysis of the data shows that none of the three mice that received an infection of 2,550 larvae by skin died, while all the mice which received an infection of 2,150 to 2,550 larvae by mouth died within 7 days. This difference is probably due to the failure of some of the larvae to penetrate the skin since equal oppportunities for penetration are not present in the two methods. It is assumed, for
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The purposes of this work, that 3,000 larvae given by either route of infection is the number of larvae that approximates the average lethal dose; i.e., it will kill a mouse within 7 days after infection.

Counts were made of the larvae recoverable from the lungs of animals that died from the infections. These counts showed no consistency and in the later work gave no indication that resistance was present; so they are not included in the data presented here.

Having established a method for testing resistance, a series of experiments was set up in order to determine whether single and repeated previous infections will induce a resistance to a lethal test infection. In order to avoid the danger of giving an animal a test infection to which it would normally be resistant, a dose greater than the approximate average lethal dose was given as a test infection. Therefore, doses of 4,000 or more larvae, which were always lethal when given by mouth and almost always lethal when given by skin, were used in all except the first experiment.

Experiment 1. A preliminary experiment with four mice (no. 4, 5, 24, and 26; see table 1) which had survived the skin infection given them in determining the average lethal dose and with three previously uninfected mice (no. 35, 36, and 37), was carried out to determine whether previous infections would confer an immunity. The four mice were each given three infections by skin during a period of 33 days and the three mice were each given two infections by skin within

### Table 2.

Data of experiment 1 showing the resistance to a lethal dose conferred on mice by previous infections with the dog hookworm, Ancylostoma caninum.

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>1st infection</th>
<th>2nd infection</th>
<th>3rd infection</th>
<th>Test infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of larvae given</td>
<td>Days after first infection</td>
<td>Number of larvae given</td>
<td>Days after second infection</td>
</tr>
<tr>
<td>4</td>
<td>1,000</td>
<td>25</td>
<td>740</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>2,100</td>
<td>25</td>
<td>740</td>
<td>8</td>
</tr>
<tr>
<td>24</td>
<td>2,550</td>
<td>25</td>
<td>740</td>
<td>8</td>
</tr>
<tr>
<td>26</td>
<td>2,550</td>
<td>25</td>
<td>740</td>
<td>8</td>
</tr>
<tr>
<td>35</td>
<td>740</td>
<td>8</td>
<td>675</td>
<td>Omitted</td>
</tr>
<tr>
<td>36</td>
<td>740</td>
<td>8</td>
<td>675</td>
<td>Omitted</td>
</tr>
<tr>
<td>37</td>
<td>740</td>
<td>8</td>
<td>675</td>
<td>Omitted</td>
</tr>
</tbody>
</table>

Controls 38, 39, 40, 49, and 50 (see table 1) died 3, 6, 15, 7, 5, and 5 days after infection.
a period of 8 days. These seven mice with five previously uninfected controls were each given the approximate average lethal dose as a test infection 7 to 14 days after their last previous infection. The results are presented in table 2. Two of the seven previously infected mice died 3 and 8 days, respectively, after the test infection and the remaining five mice survived. All five control mice died. The survival of five of the previously infected mice suggested that a resistance was acquired through previous infections.

Experiment 2. In order to demonstrate more definitely that a resistance could be induced by previous infection and to study further aspects of this resistance, an attempt was made in this experiment to do the following: first, to set up a standard method for inducing a resistance by utilizing two rates of infection; secondly, to determine how large a lethal dose was necessary to break the resistance; thirdly, to determine whether resistance could be incited through a series of mouth infections as well as skin infections; and fourthly, to test whether previous infections through the skin would render the animals resistant to test infections given by mouth. The same rate of infection was used in parts a, b, and d; in a the infections were given by skin, in b by mouth, and in d some by skin and some by mouth. In part c the sublethal infections were given by skin and the test infections by mouth.

a. Infections by skin. Seven mice were each given a series of four sublethal infections by skin at weekly intervals, at first with 250 larvae and then with double the preceding dose each time until the last dose was 2,000 larvae (250, 500, 1,000 and 2,000). A week after the last sublethal dose, a series of test infections by skin of 4,000, 8,000, and 16,000 larvae was given to these mice at weekly intervals. A check on the lethality of the test doses was made on the previously uninfected control mice. Two of the seven previously infected mice died from the test infection of 4,000 larvae. The remaining five mice resisted the test infections of 4,000 and 8,000 larvae. Two of these five resisted 16,000 larvae and two succumbed 1 and 11 days later, respectively. The fifth mouse died at the time the anesthetic was given for the third test infection. The two control mice which were infected with 4,000 larvae survived to be reinoculated the following week with 8,000 larvae. One of these mice died 4 days later, the other was given 16,000 larvae, to which infection it succumbed. One control mouse was given an infection of 8,000 larvae; and another control mouse was given 16,000 larvae. They both died as a result of their infections.

The failure of two of the control mice to succumb to the lethal
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infection even when 8,000 larvae were administered indicated that the
test infections applied by skin do not always kill the mice, although
all nine mice which received between 2,900 and 4,000 larvae (table 1)
in determining the approximate average lethal dose had died. Be-
cause of this difficulty and because the same test infection by mouth
always killed the mice, it was decided to use mouth infections to test
the resistance in all subsequent experiments.

b. Infections by mouth. Nine mice were each given a series of
sublethal infections by mouth at weekly intervals with 250, 500, 1,000,
and 2,000 larvae, the same doses used in part a. A week after the last
sublethal dose, a series of test infections by mouth of 4,000, 8,000, and
16,000 larvae were given at weekly intervals. Four of the nine mice
survived all the infections; four mice died following the infection
with 16,000 larvae; and one mouse died after the infection with 4,000
larvae. Two previously uninfected control mice were infected each
time a lethal infection was given (4,000, 8,000, and 16,000 larvae);
these all died in two to 10 days after infection.

c. Sublethal infections by skin, test infections by mouth. Eight
mice were each subjected to a series of sublethal infections by skin at
weekly intervals. In the first infection 500 larvae were administered
and 250 were added to this number at each subsequent infection until
doses of 2,000 larvae were reached (500, 750, 1,000, 1,250, 1,500, 1,750,
and 2,000 larvae). For the three test infections, which were given by
mouth in the 3 subsequent weeks, the number of larvae was doubled
each time (4,000, 8,000, and 16,000 larvae). The eight mice survived
all these test infections. Two control mice were infected each time a
lethal test infection was given. These all died within 2 days after
the larvae were administered.

d. Infection of two series of mice, one by skin and the other by
mouth. After the demonstration that a series of infections rendered
mice resistant to lethal infections, two groups of mice were treated
similarly in order to make them resistant and consequently, adapta-
ble for studies on cellular response to an infection. One of these two
groups, consisting of eight mice, was given the sublethal infections of
250, 500, 1,000, and 2,000 larvae by skin at weekly intervals. A week
after the last sublethal infection the resistance was tested with a dose
of 4,000 larvae by skin infection. The other group, consisting of
thirty-two mice, was given the same sublethal infections by mouth and
a week later received the same test infection by mouth. All of these
mice survived the test infection.
Experiment 3. This experiment was carried out in an attempt to determine whether the resistance is of long or short duration, a standard method for producing resistance having been set up. Thirteen mice were subjected to the series of four sublethal infections by mouth of 250, 500, 1,000, and 2,000 larvae at weekly intervals. The following week the resistance was tested with an infection of 4,000 larvae by mouth which all the mice survived. Forty-eight days later these thirteen mice and three previously uninfected control mice were each given an infection by mouth of 4,200 larvae. Seven of the thirteen previously infected mice survived this test infection. The remaining six mice and the three controls died 2 to 8 days later. The resistance was evidently waning since approximately half of the previously infected mice died from the second test infection.

Experiment 4. This experiment was designed to test whether a single infection would induce a resistance to a subsequent lethal infection. Eleven mice were divided into two groups, one group of six mice was infected by mouth with 1,500 larvae. Twenty-eight days later these six and the other group of five mice were each given 4,000 larvae by mouth. All five of the control mice were killed by the test infection, four on the third day and one on the fourth day after infection; all of the previously infected mice survived. These results indicate that a single infection of 1,500 larvae confers a resistance to the lethal test infection of 4,000 larvae.

Experiment 5. In order to determine whether a very low grade infection would cause the development of resistance, two groups of five mice each were infected by mouth in the following manner: The first group received five infections of approximately 50 larvae each, an average of 251 larvae being given to each of the five mice; The second group, consisting of five mice, received a single infection of 275 larvae each at the time the mice in the first group received their third infection. Both groups of mice and three previously uninfected control mice were infected with 4,000 larvae by mouth a week after the first group received its fifth infection and three weeks after the second group received its single previous infection. Three mice in the first group, two mice in the second group, and all three control mice died 3 to 7 days after infection. These results indicate that an infection of as few as 250 to 275 larvae induced some resistance to the lethal test dose of 4,000 larvae. It apparently made no difference in the production of the resistance whether this sublethal dose was given in a series of infections or in a single infection.
Discussion. This series of experiments shows conclusively that previous infection by either skin or mouth with larvae of the dog hookworm, *Ancylostoma caninum*, will induce a resistance in mice. Seventy-six of eighty-four mice subjected to previous infection survived the test infection of 4,000 larvae. Seventeen of the nineteen control mice were killed by the same test infection. The two control mice which survived were infected by skin, the less satisfactory of the two methods of infection. The immunity which developed in experiment 2 (a, b, and c) was sufficient to permit fourteen of the twenty-five mice used to survive a test infection of 16,000 larvae, which is more than five times the approximate average lethal dose.

Since the adults of this species of nematode do not develop in the mouse, this resistance is induced entirely through the reaction between the host and the larval stages, which is the period of the life cycle when there is intimate contact with host tissue. Evidence on the nature of this reaction will be brought out in a subsequent section. The immunity created by previous infection is apparently general. Sarles (1932) found that rabbits were resistant to mouth infection with *Trichostrongylus calcaratus* when previously infected by skin and to infections by skin when previously infected by mouth. In one experiment (2 c), which has been presented here, similar results have been found. In this experiment the immunizing infections were given by skin and the test infections by mouth. The mice were just as resistant to the test infections given by this method as to those given through the same route as the immunizing infections (2 a, b, d).

It is interesting to note that a single sublethal infection of 1,500 larvae induced a resistance to a lethal infection when the two infections were given at least 3 weeks apart. Likewise, a single infection of 275 larvae induced some resistance, since three of the five mice survived the lethal test dose of 4,000 larvae. A series of infections (250 larvae given in five equal infections) was no more effective in producing a resistance than the single low grade infection.

A number of problems are suggested from these results. The most important of these is the duration of the immunity. Data from experiment 4 indicate that it is of short duration, for six of the thirteen mice infected with 4,200 larvae 48 days after they had been shown to be resistant died. This needs verification and amplification. Another problem is whether a higher degree of immunity can be induced through a longer series of sublethal infections. The results of experiment 2, c, in which the longest series of sublethal infections was given, indicate that a higher degree of resistance was induced since
all of the previously infected mice survived the test dose of 16,000 larvae. It is not known how many larvae would be required to overwhelm the immunity thus produced.

Comparison of the cellular response to infection with the dog hookworm in resistant and non-resistant mice.

Taliaferro (1934) has made the statement that in the series of investigations on acquired immunity in helminth and other metazoon parasitic infections, no adequate study of its essential cellular basis has been made. He also has pointed out that the general pathological studies of such infections indicate that marked local cellular reactions occur. Stumberg (1932) followed histologically an infection of Ancylostoma caninum in the skin of dogs treated in several ways and attempted to correlate the response with local immunity. He studied sections of skin taken from four dogs which had been subjected to repeated infections through the same area of skin, one dog into which hookworm antigen had been subcutaneously injected and larvae applied to the same areas, three dogs into which antihookworm rabbit serum had been injected and larvae applied, and one dog into which heat-killed larvae were injected. He concluded from this study that there was no relation between the degree of response as related to acquired immunity and the experimental procedure.

The usual point of entry of hookworm larvae is through hair follicles, when these are present, or between the crevices formed by the scaling of the corium (Looss, 1911). Having once penetrated, they can mechanically work their way into the other layers of the skin. When large numbers of larvae penetrate there may normally be considerable destruction of the epidermis with hemorrhage, exudation and the formation of a scab. Microscopical examinations (Hoeppli, 1927) of skin from such infections have shown that there is almost complete destruction of the epidermis, which has been replaced by a thick organizing fibrinous coat containing polymorphonuclear leucocytes. In the dermis and subcutaneous tissues there are islands of wandering cells consisting of numerous polymorphonuclear leucocytes, many of which are eosinophils, fibroblasts and epithelioid cells. Stumberg has stated that the proliferation of fibroblasts is the usual cellular reaction to the injury caused by the penetration of the hookworm larvae and that the leucocytic reaction is associated only with the presence of dead larvae.

Pathological studies on parasitic nematode infections indicate that marked local cellular reactions occur. It has been shown by Hoeppli...
(l. c.) that these reactions are similar in their main features. In general they consist of a polymorphonuclear infiltration followed by the appearance of fibroblasts and later encapsulation of the worm. Upon the death of the worm, calcification or destruction by giant cells ensue. The more recent pathological studies made by O'Connor and Hulse (1932), O'Connor (1932), and Faust (1935) all agree with the main features described by Hoeppli. The normal cellular reaction to nematodes is, then, that of an acute inflammation, followed by that of a chronic inflammation and foreign body reaction.

The ability to produce easily a resistance in mice to the dog hookworm, as reported in the preceding section, provided an opportunity to study the essential cellular basis of resistance, and to determine if it is related to the pathological pictures described. This has been done by comparing the reaction to an infection at intervals of 2 to 120 hours in forty resistant and thirty-five non-resistant mice. Tissue taken from one resistant mouse 32 days after infection was also studied.

Lesions in the lungs and liver. When the mice were autopsied, a striking difference in the degree of hemorrhage in the lungs of the resistant and non-resistant mice was noted. This difference appeared only in those animals killed 24 or more hours after infection. It was always possible to distinguish the resistant from the non-resistant animal by the amount of hemorrhage. Scattered petechiae or the involvement of a portion of a lobe was the characteristic picture in the resistant mice; while the lungs of the non-resistant mice were frequently full of blood indicating that many larvae had passed through them. Of the seventy-four mice used in these studies, sections were made of the lungs of sixteen resistant and fifteen non-resistant mice. Larvae were found in the lungs as early as 12 hours and as late as 120 hours after infection in both resistant and non-resistant mice. Although no difference in the number of larvae could be detected in the sections, the extensive hemorrhage (plate I, fig. 2) in the non-resistant mice indicated that many more larvae must have passed into the capillary bed of that organ. The reduced number of larvae passing into the lungs resulted in the survival of the resistant mice when infections were given which were lethal to the non-resistant mice.

If there had been any trapping of the larvae in the resistant mice by a cellular response in the lungs, such reaction would have been seen about some of the many larvae observed. However, no reaction other than that attributable to the injury caused by the larvae passing into the capillary bed of the lung tissue was found. Edema, hemor-
rhage, serofibrinous exudate and the infiltration of a few polymorphonuclear leucocytes were characteristic of the earlier stages of the infections (12 to 48 hours) in both resistant and non-resistant mice (plate I, fig. 1 and 2). The condition is known as verminous pneumonia, and has been adequately described for ascaris, strongyloides and nippostrongylus (Hoeppli, 1927; Faust, 1935; Porter, 1935, b). Following this acute stage there was resolution in those animals which did not die. The resolution was accompanied by a thickening of the alveolar epithelium, the cells assuming an almost cuboidal shape, and by the appearance of monocytic wandering cells about the walls of the bronchi.

The deposits of pigment which Porter (1935, b) described in the lungs of rats infected with Nippostrongylus muris were also noted in this study, though they were not consistently present. Dr. Porter examined several of my slides and I have examined his. We are agreed that the microscopical appearance of the pigment is the same in both sets of slides.

These observations indicate that there is no retention of the hookworm larvae in the lungs of the mice and suggest that this organ plays no rôle in the phenomenon of resistance. They suggest, rather, that the host response must act upon the larvae before they reach the lungs, since the severity of the lesion is dependent upon the number of larvae breaking out of the lung capillaries.

No explanation for the mechanism of resistance having been found in the lungs, other tissues in the normal path of the larvae were examined. The liver was studied next, for when infections are given by mouth the larvae pass through the capillary bed of this organ on their way to the heart and lungs. Since it is an organ richly endowed with the components of the reticulo-endothelial system, the liver might be an "immunological filter" for nematode larvae which have to pass through it in order to complete their life cycle. The finding of ascarid larvae encapsulated and degenerating in the liver (Hoeppli, 1927) suggests that the liver plays a part in preventing the completion of the life cycle of these worms. Further evidence of the importance of the liver in immunity to parasitic infections has been presented by Taliaferro (l. c.) in his summarization of the work done by himself and co-workers on malaria. The disappearance of the malarial parasites was found to be directly correlated with the activity of the differentiated macrophages of the spleen and liver and to a less extent the bone marrow.

In the present studies no gross liver lesions were noted in either
PLATE I.

Fig. 1. Section through a larva in the lung of a resistant mouse 48 hours after infection. Note the sero-fibrinous exudate, S, about the larva. × 494.

Fig. 2. Section through the lung of non-resistant mouse 48 hours after infection. Note the edema and severe hemorrhage into the alveoli. × 124.

Fig. 3. Section through a larva in the liver showing the typical lesion. Note the red blood corpuscles, R, and the absence of necrosis of liver cells and polymorphonuclear infiltration. × 494.

Fig. 4. Section of a larva in the hypodermis of a non-resistant mouse 24 hours after infection. Note the absence of cellular infiltration. × 494.
resistant or non-resistant mice. Sections were made of the livers of fourteen resistant and eleven non-resistant mice, representing a period from 2 to 108 hours after infection. The microscopical changes in these livers were compared and found to be identical. Thus again no evidence of a cellular response which could be correlated with the resistance of the mice could be found. The typical lesion caused by the larvae is shown in figure 3 (plate 1) and is characterized by slight hemorrhage and some fatty degeneration of the liver parenchyma. As the larvae pass through the smaller capillaries they push aside the liver cells, but do not cause an apparent pressure necrosis. There was no evidence of walling off or retention of the larvae.

During the course of these studies, larvae were found incidentally in the mesenteric lymph nodes and in the mesenteries themselves. No evidence of a cellular response was noted in either of these tissues.

It has been pointed out in the previous section that mice subjected to a primary dose of 4,000 or more larvae died from verminous pneumonia produced by the larvae breaking out of the capillary bed of the lung. Previously infected mice were resistant to such a lethal dose. The studies which have just been reported show that fewer larvae reached the lungs in resistant mice, a circumstance which explains why they were not killed by the lethal dose. There was no walling off or retention of the larvae in this tissue. Likewise, no evidence that a cellular response trapped or inhibited the larvae was found in the liver of resistant mice. Therefore, if any cellular reaction is responsible for the resistance which was produced, it must occur in the tissues which the larvae enter first, e.g., in the skin and the wall of the digestive tract.

Cellular response in the skin. The histological reaction called forth by the larvae penetrating the skin will be presented first because more extensive studies have been made on this tissue than on the digestive tract. Sections were made of the skin of thirty-two mice, eighteen of which had become resistant from previous infections. All the resistant mice were survivals of experiment 2. Ten of them, used in this study in connection with the first 48 hours, were survivals of parts a, b, and c of that experiment. They had been subjected to a series of immunizing infections, some percutaneous and some per os, of either 250, 500, 1,000 and 2,000 larvae or 500, 750, 1,000, 1,250, 1,500, 1,750, and 2000 larvae at weekly intervals. Following these infections they had survived test infections of 4,000 8,000, and 16,000 larvae. The remaining eight mice were all rendered resistant through a series of infections by mouth of 250, 500, 1,000 and 2,000 larvae.
given at weekly intervals and tested with an infection of 4,000 larvae given by mouth (see experiment 2, d). Approximately a month elapsed between the last test infection and the infection of 4,000 larvae which was given to each of the eighteen mice used in these studies.

The histological changes which occurred in the skin of the non-resistant mice are described first to lay down a basis for comparison with those in resistant mice. The larvae in the non-resistant mice were found penetrating just as they do in the normal host, that is, through hair follicles and in between the crevices formed by the epidermal scales. They were found in all layers of the skin as early as 12 hours after infection and were present in the hypodermis as late as 72 hours after infection. The infections older than 72 hours were not adequately studied in this connection, to determine what happened following this time. In the few sections studied after 72 hours, only one or two larvae were found. No cellular response was noted until 18 hours after infection. At this time there was some general infiltration of polymorphonuclear neutrophilic leucocytes, which was increased 6 hours later. This was most pronounced where the epidermis had been damaged by the penetration of the larvae. However, at this time there was still no infiltration about many of the larvae (plate I, fig. 4). Thirty-two to thirty-six hours after infection the infiltration had reached its height. It was not intense but it was marked. Following this time the character of the reaction changed. Forty-eight hours after infection a round cell infiltration had occurred, as is illustrated in figure 7 (plate II). The number of polymorphonuclear cells was reduced, many showing pyknotic nuclei, and monocytes and cells which appeared to be fibroblasts were present. There was also moderate edema (compare fig. 4 with fig. 7). Seventy-two hours after infection the edema and marked polymorphonuclear infiltration were gone, a circumstance indicating that the reaction was definitely waning. Fibrous tissue was noticeably increased. The total pathological picture, that is, the edema, leucocytic infiltration and presence of fibroblasts was identical to the characteristic reaction described by Stumberg in the skin of the dog. Apparently the larvae called forth the same cellular reaction in the primary infection of the abnormal host that they did in the primary infection of the normal host.

In the case of resistant mice, as in the non-resistant mice, larvae were found in all layers of the skin 12 hours after infection. The larvae seemed to enter just as easily, but once in the skin there was a marked difference in the cellular response. The polymorphonuclear
Fig. 5. Section through a larva in the subcutaneous stratum of the hypodermis 12 hours after infection in a resistant mouse. The larva is lying in a lymphoid space and is being surrounded by an infiltration of polymorphonuclear neutrophilic cells, P. H, hair follicle. × 494.

Fig. 6. Section through a larva in the fascia of the hypodermis of a resistant mouse 36 hours after infection. This illustrates a typical island of polymorphonuclear cells which surround a larva. Note also the intense infiltration of polymorphonuclear cells in the subcutaneous stratum. × 124.

Fig. 7. Section through an area of the subcutaneous stratum of the hypodermis of a non-resistant mouse 48 hours after infection. This shows the characteristic local cellular response in a non-resistant mouse at this time. Polymorphonuclear, mononuclear and fibroblastic cells are present. × 494.

Fig. 8. Section through a larva in the fascia of the hypodermis of a resistant mouse 72 hours after infection. This portion of the larva has lost its capacity for staining. Note the change in the character of the reaction, there being few polymorphonuclear and many monocytic and fibroblastic cells present. × 494.
neutrophilic cells appeared earlier, for there was a mild but noticeable infiltration of these cells 6 hours after infection. This became quite as pronounced by 12 hours after infection (plate II, fig. 5) as was found 6 hours later in the non-resistant mice. Eighteen hours after infection the infiltration of the polymorphonuclear neutrophilic cells was more extensive and the islands about the larvae in the subcutaneous tissue were striking. There was also edema. Six hours later the degree of infiltration had increased and there was seen the first tendency for the leucocytes to gather about larvae still remaining in the hair follicles.

Necrosis and sloughing of the epidermis with the formation of a scabby excrescence containing polymorphonuclear cells was noted for the first time at 28 hours after infection. This was general over the surface of the skin, while in the non-resistant mice it could be found only in minute areas.

A slight change in the type of cells occurred in the resistant mice by 32 hours after infection. The infiltration of leucocytes had reached its greatest intensity in both non-resistant and resistant mice at this time. However, the difference between the resistant and non-resistant mice was more striking than heretofore. Both the general leucocytic infiltration and the concentration of the leucocytes around the larvae were more pronounced in the resistant mice (plate II, fig. 6), while in the non-resistant mice the local areas of infiltration (plate II, fig. 7) were not as large and rarely contained a larva, seeming only to mark the paths the larvae had taken in migrating through the tissues. At this same time mononuclear cells were first noted in the resistant animals.

The change in the picture from that of an acute inflammation to that of a chronic inflammation was becoming more apparent in the resistant mice 48 hours after infection. During the next 24 hours the polymorphonuclear cells gradually became more degenerate and fewer in number and the lymphocytes, macrophages and fibroblasts became the predominating cells. While this was occurring in the resistant mice, the reaction was waning in the non-resistant mice. Figure 8 (plate II) shows a larva in the fascia of the hypodermis of a resistant mouse 72 hours after infection, illustrating the relative absence of polymorphonuclear cells, the presence of macrophages immediately about the larva, and an outer layer of fibroblasts. This particular larva had lost its capacity for staining in about one-half of its length, the other half seeming to stain normally; apparently the larva was dying (Stumberg, 1932). During this period (48 to
72 hours after infection), the necrosis, sloughing and scabby excrescence were pronounced in the resistant mice.

The further progress of the reaction in the resistant mice was shown at 96 and 120 hours after infection, when mononuclear cells having an epithelioid character and giant cells were found in the area immediately about a larva. This is illustrated in figure 9 (plate III). Several larvae in similar islands of cells were stained poorly, a reaction suggesting that they were dying or dead. The appearance of giant cells adds to the reaction, the character of a foreign-body response. Further illustration of this may be seen in figure 10 (plate III). In this section, taken 32 days after infection, a larva is shown immediately surrounded by epithelioid and giant cells, the whole area being encompassed by fibroblasts. The larva itself appears to be disintegrating due to the attack of these cells. However, it was impossible to determine whether the host cells were actually inside the larvae or merely on its outer surface. The dense superficial exudate was still pronounced at 96 hours and was found 32 days after infection. In the latter, organization had taken place and a new layer of epidermis had grown over the dermis.

Mention should be made here that the above reactions were noted about one larva in one of the non-resistant mice and that all of the larvae found in the resistant mice were not surrounded by an area of reaction. One larva, found in the skin of a resistant mouse taken 32 days after infection, was free from any cellular response and was well-stained; this suggests that it was still alive at the time the tissue was fixed.

Cellular response in the esophagus and stomach. A cellular response definitely related to the resistance having been found in the skin of resistant mice, it was of interest to determine whether the same reaction occurred in the tissues of the alimentary canal, the point of penetration when infection was given by mouth. All ten of the resistant mice used in this phase of the study had been subjected to a series of immunizing infections by skin of 250, 500, 1,000, and 2,000 larvae given at weekly intervals (experiment 2, d). A week after the last immunizing infection the resistance was tested with the lethal dose of 4,000 larvae again given by skin. Approximately a month elapsed between this test dose and the infection of 4,000 larvae given to each mouse by mouth for this study. A survey of the sections indicated that many larvae from this infection had penetrated through the lower esophagus and stomach. A few larvae were found entering the tissues of the portion of the duodenum examined. Since
PLATE III.

Fig. 9. Section through a larva in the fascia of the hypodermis of a resistant mouse 96 hours after infection. The larva is surrounded by mononuclear cells which are beginning to have the appearance of epithelioid cells, a few polymorphonuclear cells with pycnotic nuclei, and a giant cell, G. × 494.

Fig. 10. Section through a larva in the hypodermis of a resistant mouse 32 days after infection. Note the epithelioid cells, the giant cell, G, and the fibroblasts. The nuclei of this larva still stain but they appear pycnotic. ×494.

Fig. 11. Section of larva, L, in the submucosa of the stomach of a resistant mouse 48 hours after infection. The larva is lying just beneath the muscularis mucosa in a nest of polymorphonuclear cells. Note the sero-fibrinous exudate, S, in the mucosa to the top. × 494.

Fig. 12. Section of a larva in the submucosa of the stomach of a non-resistant mouse 48 hours after infection. Note that there is little cellular infiltration. × 494.
sufficient larvae were found in the tissues of the esophagus and stomach to determine the nature of the cellular response, the detailed study was limited to these organs. The seventeen mice (ten resistant and seven non-resistant) were killed at intervals of 12 hours over a period of 5 days.

The reaction to the infection of the seven non-resistant mice will be described first. No infiltration of cells was noted 12 hours after infection. Larvae were present in all layers of tissue in both esophagus and stomach. Twenty-four hours after infection a mild general infiltration of polymorphonuclear neutrophilic cells was present, which in particular marked the migration paths of the larvae. No change was noted 12 hours later. Forty-eight hours after infection there was a somewhat heavier infiltration. Figures 12 and 14 (plates III and IV) show the usual degree of infiltration of polymorphonuclear cells, which is not as extensive as that in the skin of non-resistant mice (see plate II, fig. 7). From this time on the reaction grew less, so that no infiltration of leucocytes was found about larvae at 120 hours after infection. In general, then, except for minor variations, the reaction to the primary infection in the wall of the esophagus and stomach was the same as that in the primary infection in the skin.

In the resistant mice only a slight general infiltration of polymorphonuclear neutrophilic cells was noted at 12 hours after infection. This had increased somewhat by 24 hours after infection; edema and dilatation of the capillaries were also seen. Larvae surrounded by islands of cells were not seen until 48 hours after infection. However, the nature of the reaction observed at this time (plate III, fig. 11) indicated that it must have been going on for some time, for it consisted not only of polymorphonuclear cells but also of some round cell infiltration. Considerable edema and fibrin were also present. Polymorphonuclear cells were commonly seen in the lumen of both organs. This is in contrast to the condition seen at this time in the non-resistant mouse, in which only a mild polymorphonuclear infiltration was present (plate III, fig. 11 and 12; plate IV, fig. 13 and 14). Seventy-two hours after infection the reaction in the resistant mice was assuming the character of a chronic inflammation. Figure 15 (plate IV) illustrates this, showing an island of cells made up of many polymorphonuclear cells with pyenotic nuclei and macrophages about a larva lying in the submucosa of the esophagus. In addition, there was noted in the resistant mouse a necrosis and sloughing of the mucosa and the formation of a pseudodiphtheritic membrane consisting of sloughed epithelial cells, polymorphonuclear neutrophils.
and a serofibrinous exudate. This condition was not present in the non-resistant mouse.

Giant cells (plate IV, fig. 16) were found 96 hours after infection in the resistant mice. The majority of the polymorphonuclear cells were obviously degenerate and the monocytic cells showed some of the characters of epithelioid cells. Fibroblasts surrounded the area. Thus again, as in the skin, it was noted that the terminal response was a foreign body reaction. The pseudodiphtheritic membrane was present at this stage also.

One larva was found 60 hours after infection in an area of chronic inflammation in the submucosa of the alimentary canal (stomach) of a non-resistant mouse just as one was found in the skin of a non-resistant mouse. Also, not all of the larvae found in the resistant mice were surrounded by a cellular reaction. It may well be that the larvae found surrounded by a cellular reaction in the non-resistant mice had a lower vitality than the other larvae so that they were unable to overcome the tissue barriers and were trapped by the infiltrating cells.

**DISCUSSION.**

The foregoing observations show that there is a definite relationship in mice between the cellular response and resistance to infection with the dog hookworm. The polymorphonuclear infiltration followed first by macrophages and then by epithelioid cells and giant cells with the whole area encompassed by fibroblasts is a common histological change occurring about nematodes, but hitherto its relationship to resistance has not been demonstrated. It has been shown here that this response occurs much more intensively in resistant mice than it does in non-resistant mice. Apparently previous infection influences the host in some way so that there is cellular infiltration which surrounds the larvae in the area of penetration.

The activity of polymorphonuclear leucocytes and macrophages in the resistance just reported is similar in its main features to the local response which has been described about other nematodes which have a phase of intimate contact with the host tissue. It is suggested, therefore, that this activity may be the chief mechanism of resistance to all nematodes found in the tissues. Taliaferro and his coworkers believe that this mechanism is of prime importance in the production of host resistance to protozoan infections, as has been shown by relating the activity of macrophages to the disappearance of malarial organisms from the peripheral blood (Taliaferro, 1932) and the effect
Fig. 13. Section through the esophagus of a resistant mouse 48 hours after infection. Compare this with figure 14, noting the difference in degree of cellular response. X 124.

Fig. 14. Section of a larva in the esophagus of a non-resistant mouse 48 hours after infection. Note the absence of a reaction in comparison with the preceding figure. X 124.

Fig. 15. Section through the esophagus of a resistant mouse 72 hours after infection, showing a larva in the submucosa surrounded by an island of cells. X 124.

Fig. 16. Section through a larva in the submucosa of the stomach of a resistant mouse 96 hours after infection. Note that the larva is surrounded by polymorphonuclear cells with pyecnotic nuclei and epithelioid cells. Note also the giant cell, G. X 494.
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Of splenectomy and blockading in trypanosome infections (Taliaferro et al, 1931; Taliaferro and Pavlinova, 1936). Taliaferro's findings with protozoa, the relationship demonstrated here in nematode infections, and the importance of macrophages in bacterial immunity (Gay, 1935), all point to a unity in the defense mechanism against invading organisms.

This definite cellular response in the tissues must also be related to some of the four expressions of resistance mentioned in the introduction of this paper. It is the author's belief that the intimate contact between host tissue and parasite affects particularly the number of worms developing, thus reducing the worm burden, and to a lesser degree the rate of development of the worms. The adult stage in the intestine apparently affects only the egg production of a superinfection as has been shown by Spindler (1936). After he had introduced adults and pre-adults of nippostrongylus into rats by means of a duodenal tube, he later gave the animals a percutaneous superinfection; the resistance which was present only affected the egg production of the worms developing from the cutaneous infection. There was no evidence that the rate of growth was affected or that fewer worms developed as has been shown by Porter (1935, a) in rats and mice previously infected by skin. This evidence along with the results presented in this paper show that the phase of intimate contact between host tissue and parasite should be separated from the adult phase in the intestine in an analysis of the host-parasite relationships in acquired resistance.

The resistance reported here, while appearing in a local tissue, is apparently general in nature since the reaction occurs where no previous infection has taken place through that tissue. This is opposed to the view of Chandler (1935) who stated that the resistance of rats to nippostrongylus was a property of the intestinal mucosa. Chandler arrived at this conclusion because he was unable to passively transfer the resistance in the serum or to find any resistance passed from an infected member to the uninfected member of parabiotic twin rats.

The evidence in support of our view on the general character of this resistance follows. Sarles (1932) found that rabbits previously infected either by mouth or by skin with Trichostrongylus colubratus were resistant to subsequent infections by either method. In the preceding section the results of one experiment (2, c) showed similarly that when the immunizing infections of hookworm larvae were given by skin the mice survived test doses given by mouth. In addition,
there is the evidence derived from the observations that the cellular response is the same in all the resistant mice even though the immunizing infections were given through a portal of entry different from that infection followed histologically. Eleven of the sixteen resistant mice used in the studies on the cellular response in the skin received all of their previous infections by way of the mouth. All of the resistant mice used in the studies on the cellular response in the esophagus and stomach received all of their previous infections through the skin. If this resistance were entirely local, the reactions described would have been found only in the skin when previous infection took place in the skin, or likewise, only in the wall of the alimentary canal when previous infection occurred through those tissues. It is local, however, in that it is present only in those tissues which form the first barrier for the larvae and not in other tissues which lie in the normal course of the migration of the larvae, that is, the liver and lungs.

The polymorphonuclear infiltration in the skin and wall of the digestive tract of the mice was marked by the absence of eosinophils. A careful search was made for this type of cell but none was found. Undoubtedly some eosinophilic cells were present, but only occasionally. They were not present in groups as has been found in the skin of dogs by Hoepli (1. c.) with *Uncinaria stenocephala* and *Strongyloides stercoralis* and by Faust (1935) with strongyloides.

The edema, necrosis and sloughing of the epidermis, and the scabby excrescences found on the surface of the skin of the resistant mice have also been found by Faust (1935) following the penetration of strongyloides in the skin of dogs and by Sarles (1929) in the skin of old dogs following the penetration of hookworm larvae. Apparently, this reaction has been attributed to the mechanical irritation of the larvae alone. Since a similar reaction has been observed in the alimentary canal and the reaction is much more pronounced in previously infected mice than in mice that have never before been infected, it cannot be entirely the result of mechanical irritation but is magnified by a hypersensitivity. Hypersensitivity to hookworm in man, regardless of the presence or absence of concurrent infection, is known through the work of Coventry and Taliaferro (1928). Definite proof or disproof of this remains for future work.

The observations which have been made here and by Stumberg (1932), Faust and Kagy (1933), and Faust (1933 and 1935) lead to the conclusion that there is a definite cutaneous retention of larvae of nematodes which penetrate the skin. Mention has been made of the
one larva, found in the skin of a resistant mouse 32 days after infection, which had not lost any of its capacity for staining. My observations of the larvae in the esophagus and stomach suggest that there may likewise be retention of larvae in these tissues. In this connection the statement of Schwartz, Alicata and Lucker (1931), who found that many of the worms from a second infection of nippostrongylus were still in the lungs 13 to 16 days after superinfection, has apparently confused several authors. Graham (1934) and Spindler (1936) have stated that these larvae were retained in the lungs in a state of inhibited development. Porter (1935a) failed to confirm this retention. It seems to me that the explanation for these findings lies in the fact that there was a cutaneous retention of the larvae and that some of them finally escaped from the skin and migrated to the lungs.

Since the immunity reported here is dependent on the activity of the polymorphonuclear leucocytes and macrophages, there must be some antibody present which stimulates the pouring forth of these cells into the invaded tissues. It is evident that this result is conditioned by previous infection, since in resistant mice the response is much earlier (6 hours after infection) and is much more intensive and extensive than in the case of primary infection (18 hours after infection). In addition, the fact that the same response is present in the resistant mice no matter what portal of entry has been used for the previous infection shows that the immunity is general. It is, therefore, suggested that this local cellular response is dependent upon some humoral antibody.

The suggestions made here (1) that the activity of the polymorphonuclear cells and macrophages is the chief mechanism of immunity to nematodes, (2) the dependence of this activity on a humoral antibody, and (3) the presence of a hypersensitivity need to be studied further. They should be checked by studies involving the normal host-parasite relationships in both the dog hookworm and other nematode parasites.

**Summary.**

Studies were carried out using mice as experimental hosts to determine whether a true acquired immunity can be established against the dog hookworm, *Ancylostoma caninum*. The use of an abnormal host, such as the mouse, is advantageous because the larval stages pass through the tissues in the normal manner but the parasites are unable to establish themselves in the intestine. This makes it possible to de-
termine the importance in the development of immunity of the larval phase of the life cycle where intimate contact between the parasite and host tissue occurs.

It has been established that 3,000 hookworm larvae given by either skin or mouth is an approximate average lethal dose for mice, usually killing them within 7 days after infection by producing verminous pneumonia.

It was found that a series of sublethal infections given at weekly intervals induced a resistance to an otherwise lethal infection of larvae. The resistance was produced equally well by either skin or mouth infections and was great enough to permit most of the animals in certain experiments to survive a test infection of more than five times the average lethal dose. The resistance induced by a series of skin infections was just as effective against lethal infections given by mouth as that induced by a series of mouth infections, a circumstance indicating that the resistance was general. It was also of relatively short duration, since half of the resistant mice in one experiment survived a test infection given 48 days after the last previous infection. A single infection produced a resistance; further, a series of very low grade infections and a single low grade infection produced a slight degree of resistance.

The cellular reactions to a test infection of 4,000 larvae were followed in nonresistant mice and in mice previously subjected to a series of sublethal infections which rendered them resistant to a lethal test infection. By comparing sections of the lungs of the resistant and non-resistant mice after the test infection, a marked difference in the extent of the lesions was found. It was noted that the death of the non-resistant mice was caused by a greater number of larvae breaking out of the capillary bed of the lung. No retention or walling off of the larvae was found in the lungs or liver of either the resistant or non-resistant mice. A definite reaction was found in the skin and wall of the esophagus and stomach, the first tissue barriers through which the larvae must penetrate. In both resistant and non-resistant mice this retention began with an infiltration of polymorphonuclear neutrophilic cells. In the resistant mice this infiltration started sooner and was more extensive and intensive than in the non-resistant mice. Also in the resistant mice the leucocytes seemed to have a greater affinity for the larvae because marked islands of cells were formed about larvae. At 48 hours after infection the character of the reaction about the larvae was changing to that of a chronic inflammation in the resistant mice and was starting to disappear in the
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non-resistant mice. At this time in the resistant mice there was a considerable necrosis and sloughing of the epidermis and mucosa and also a scab on the skin and a pseudodiphtheritic membrane in the esophagus and stomach which was interpreted as due to a hypersensitivity. Seventy-two hours after infection the reaction had almost disappeared in the non-resistant mice leaving only an increase in fibroblasts, while in the resistant mice the destruction of the epidermis was still apparent and the islands of cells about the larvae had all the characteristics of chronic inflammation. Giant cells were found in the resistant mice at 96 hours after infection, giving to the reaction around the larvae the appearance of a foreign-body response. A chronic inflammation was found about two larvae in two non-resistant mice. However, the much greater frequency of the reaction in the resistant mice led to the conclusion that it was related to the resistance, and would, therefore, explain the ability of the previously infected mice to resist infections which were lethal to the controls. This is apparently the first time that this well-known histological reaction has been experimentally related to resistance to nematodes.

It seems altogether possible that larvae are retained only in the first tissue barriers encountered, in this case the skin and wall of the alimentary canal. Those found in the lung long after infection probably have only recently escaped from these primary tissue barriers.

It has been suggested that this type of cellular response may be the mechanism of resistance to all nematodes which invade host tissues. Since the immunity occurs in a local tissue but is stimulated by infection in an entirely different tissue, it is considered general. Therefore, the presence of a humoral antibody has been suggested.

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