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STUDY OF BIOLOGICAL PROPERTIES OF *TRICHINELLA SPIRALIS* NEWBORN LARVAE AND THE ANTIPARASITIC MUCOSAL IMMUNITY OF THE HOST

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1. ABSTRACT

Studies of biological properties of newborn larvae of *Trichinella spiralis* and immune responses against newborn larvae in the host are reviewed. The biological properties of newborn larvae examined are the natural production, migration, surface antigens and their maturity and effects on the host. Various mechanisms of host immunity to newborn larvae are reviewed, including the stage specificity, kinetics, cell-mediated vs. antibody-dependent immunity in the mucosal and other tissues of the host.

2. INTRODUCTION

Trichinella spiralis (*T. spiralis*) is a unique nematode parasite that spends its larval and adult life in the same host. It may infect almost any mammal, although humans, swine and rats are medically more important hosts (1-4). Microscopic newborn larvae (NBL)are produced by the female adult worms in the small intestinal wall and they traverse the mucosal tissues in the intestine and disseminate systemically in the host, causing tissue damage and occasionally death. Acquired resistance to *T spiralis* infections has been recognized for more than 60 years(5). It has been demonstrated that every stage of the life cycle of *T. spiralis* can evoke a protective host immune response during infection(6-15)and each response is stage-specific due to the uniqueness in both the cuticular antigens and the excretion-secretion antigens of each stage. NBL have been recognized to induce a strong immune response in rodents and pigs(16-20). Although many biological properties of NBL have not been well understood, immunity has been demonstrated *in vivo* using immunization with NBL followed by stage-specific challenge. A great deal of *in vitro* work has focused attention on the role of the antibody mediated granulocytic killing of the larval worms.

The effectiveness of immunity to NBL *in vivo* has been questioned on the belief that it occurs too late to exert an effect during the primary infection and, in a

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secondary infection, worm rejection usually occurs before NBL are produced. This view may be true for some rodents capable of early strong expulsion of adult worms in a primary infection, e.g., NIH, NFS, NFR mice and most inbred rats. However, in a primary infection, the period of residence of adult worms in the intestine is variable depending on host species, e.g., the pig and some inbred mice reject adult worms quite slowly (20,21), and it also depends on the genotype of hosts within a given species (21-23) as well as variables such as infection dose (24,25). Thus, if a challenge infection is sufficiently delayed after the primary infection, adult worms will develop and persist long enough to produce NBL. These observations suggest that protective immunity to NBL cannot be discounted a priori especially as the *in vivo* expression of anti-NBL immunity has not been assessed experimentally during natural primary or challenge infections. The following report reviews the literature and presents recent data from this and other investigators that address these questions.

3. BIOLOGY OF NEWBORN LARVAE

3.1. Newborn larvae production *in vivo*

In order to study the immune response against NBL, the biological properties of NBL need to be understood. It is known that female *T. spiralis* worms are inseminated from 30-40 hr after infection (26-29) and the first NBL are shed from day 5 as shown by both *in vivo* and *in vitro* experiments (16,30). Although there have been *in vitro* studies regarding the fecundity of female worms (31,32), it may not reflect the fecundity *in vivo*. Little direct information is available concerning NBL production *in vivo* due to the inaccessibility of the female worm in the intestine. Nevertheless, attempts have been made to estimate the reproductive potential of individual females and the kinetics and reproductive potential of different sized infections.

Four methods have been employed to determine NBL production or the overall success of an infection *in vivo*. The first approach was to determine muscle larvae burdens after transplantation of single females or paired adult worms intraintestinally in rats (33,34) or mice (35,36). Only the latter provides a true estimate in a species that mates more than once. Total production per female varied from 345(34)to 1660(36). These experiments probably provide a more accurate estimate of the reproductive potential of *T. spiralis* rather than actual reproduction as it might not occur in infections of anything more than a minuscule number of worms.

A variant of this approach has been to determine eventual muscle larvae burden after a given muscle larvae infection (6,22,37-41). Total muscle larvae recovery in these experiments has often been considered to represent total NBL production *in vivo*. However, this approach is based on the assumption that all NBL produced reach and encyst in muscle tissue. Adult female worms in the intestine are often not quantitated hence the numerical base for calculating reproduction is the dose of worms given. This is not reliable in that no infection ever successfully establishes at 100% level and, numerous factors, e.g., infection dose, fecundity of females, the rejection rate of intestinal adults, the genetic influence(21,42-45), the innate resistance and specific immunity against NBL and, successful maturation of larvae in muscle can all influence the ultimate muscle larvae burden. Indeed, studies of muscle larvae burden in different inbred mouse strains given identical infections unequivocally demonstrate the strain-to-strain variations that occur in this parameter (43-45). A different method was used by Denham and Martinez (16) who administered the drug-methyridine to eliminate adult worms in the small intestine at specified intervals after initial infection. Methyridine does not affect NBL that have been produced. By examining muscle larvae burden of mice infected for 5 to 2l days before injection of methyridine the dynamics of production could be estimated. Using this method, the peak NBL production occurred 7 days after infection.

The most complete attempt to quantitate larval production and to relate this to muscle larvae burden was made by Despommier *et al.*(46). These authors combined direct *in vitro* counts of larvae produced by female worms cultured over a 24-hr period and, multiplied this by the number of female worms present in the small intestine to derive the total daily production. Cumulative estimated larvae production was found to be within 2% of total muscle larvae burden in CFW mice. Using this method, peak larvae output occurred on day 6 post infection. The same method was used by Bell *et al.*(45) with C_3H, NFR and DBA/1mice. These authors found that in C_3H strain, 13% fewer larvae were produced than were recovered as muscle larvae. In the other two strains, the total muscle larvae burden was only 68% and 62% respectively, of expected, based on *in vitro* NBL output and total intestinal female count. Muscle larvae development from a given NBL output is thus a strain specific process in mice. Furthermore in some strains, significantly fewer muscle larvae are found than there are NBL produced.

The first quantitative analysis of NBL production *in vivo* was carried out by Harley and Gallicchio(30)who counted NBL in thoracic duct lymph and various other tissues and organs of rats from 4 to 15 days after infection. Their results showed that NBL were first produced on day 5 after infection and that production peaked on day 9. In their experiments, the thoracic duct was the major outlet for larvae migrating from the intestine to the blood. Our own studies (47) followed the same format as those of Harley and Gallicchio (30) except that NBL output in portal vein

blood was quantitated and we used acute preparations (6-24 hours) for thoracic duct lymph collection. Under these conditions, NBL output in lymph peaked at day 7 but actual output was similar for the first three days of production (6, 7, 8) and dropped precipitously thereafter. The differences in kinetics and in total larvae output in Harley and Gallicchio's(30) experiments and ours may reflect an immunosuppressive effect produced by chronic drainage of the thoracic duct. Other possible influences, such as the strain of rat used, were eliminated through the use of common strains.

Analysis of total recovery of NBL in the three major *in vivo* sites of migration, the peritoneal cavity, thoracic duct lymph and blood gave numbers of NBL that were compatible with total muscle larvae burden as defined by digestion. With an infection of 4,000 muscle larvae, 10,103 NBL were recovered from peritoneal cavity, 8,238 from thoracic duct lymph, 781,200 from portal vein. The total recovery was 799,541 and the final muscle larvae burden was 615,933±159,546. Estimation of total NBL production by the *in vitro* techniques of Despommier *et al*.(46) produced a daily larval output that was kinetically very similar to our data obtained from direct *in vivo* quantitation(47). The major difference is that *in vivo* estimates of NBL output tend to be 10-20% higher than the muscle larvae burden that is found. Part of this discrepancy is due to the ability of NBL to recirculate, the net effect of which is an over estimate of NBL production *in vivo*.

3.2. Newborn larvae migration

The route undertaken by NBL in their passage from the intestine to striated muscle has been speculated on since the turn of the century. The three main routes that have been suggested are: larvae migrate via the blood circulation; migration occurs through connective tissue and body fluids; migration is from lymph to blood. Until the late sixties, the identification of NBL in cardiac blood had led to a general inference of a blood-borne migration (1,27,48-53). No direct information on the means by which larvae gained access to the blood was, however, available. Berntzen (54) and Shanta and Meerovitch (55) investigated this question and found large numbers of NBL in the mesenteries and the fluids of the peritoneal and thoracic cavities of rats and mice. They failed to find NBL in the mesenteric lymphatics, mesenteric lymph node, thymus, liver and lungs by the histological methods employed. NBL were rarely found in thick blood smears. These findings led both groups to conclude that NBL migrated from the small intestine through connective tissue and body fluid to striated muscle. Both investigations suffer from their reliance on histological sampling techniques that are inadequate for the detection of NBL(12OX10um, 56).

Dennis *et al*. (57) examined the question by quantitating NBL infectivity after their injection intraduodenally, intravenously (iv), or intraperitoneally (ip) into mice. Specimens of diaphragm muscle of these mice were examined for muscle larvae on day 14. They found muscle larvae in the diaphragms of iv and ip injected mice only. A similar finding in rats showed that 66% of the NBL injected iv matured to muscle larvae whereas only 2% of the larvae injected ip established in muscle. The first quantitative study of natural NBL migration was again that of Harley and Gallicchio (30) described above. These authors searched for, and found, NBL in every major organ examined, as well as in the peritoneal cavity and blood. Since about 70% of the NBL recovered were from fistulae established in the thoracic duct, it was suggested that the main pathway of NBL migration was from the small intestine to draining lymphatics and then to blood and muscles. None of the above studies examined or quantitated NBL migration in the hepatic portal vein that carries all the upper intestinal venous return to the heart.

Our studies addressed this question by obtaining both thoracic duct lymph and portal vein blood samples from individual rats using acute preparations. With this method lymph was collected for 6 hours after which time rats were anesthetized and peritoneal cavity and portal vein blood samples collected. By measuring portal vein blood flow, it was found that over 97% of NBL entered capillaries directly in the intestine(47). Once in the blood, larvae rapidly traverse capillary beds in the liver and lung and can reappear in the venous blood 8 minutes after their initial introduction into the venous circulation. This is the time required by the fastest larvae to complete one full passage; however, the majority of the larvae injected in one bolus return to the starting point some 20-30 minutes after their introduction(58). By injecting a known number of NBL into the circulation and sampling the blood at intervals thereafter, we determined that about 40% of the circulating population disappears every hour. Most of these presumably penetrate striated muscle. With this speed of migration, 75% of the initial population migrate away from the blood within the first 3 hours after their introduction. A small proportion continues to circulate and larvae may still be in non-muscle tissue 24 hours after their inoculation(58).

NBL, thus, migrate through the body quite extensively before they eventually gain access to muscle cells. Relatively few appear to enter the striated muscle on their first passage through this tissue as can be seen from the following calculations: larvae recirculate in about 20 minutes and total body striated muscle mass is around 40% for most mammals. Therefore 40% of NBL should be randomly delivered to muscle in the first passage; if they all penetrated and 40% of the remainder also penetrated during the next 20 minutes when they were delivered to muscle and other tissues, then 78% of the starting population would have been removed from the circulation within the first hour. This is not observed empirically. It would appear that a large number of NBL that are delivered to muscle either remain in capillaries

and pass right through the muscle or actually penetrate into the muscle tissue and then leave it and reenter the circulation. This concept is consistent with the data of Despommier *et al*. (59) who found that 40% of the muscle larvae recovered after direct injection of NBL into muscle were found in sites other than where they were injected. It should be mentioned that while the above experiments have provided the first estimation of the dynamics of larval migration via blood and tissue, they are based on the injection of a single bolus of larvae. It is possible that single bolus injection may influence migratory patterns in some way. This has not yet been examined, hence studies with small numbers of radiolabelled larvae are needed to confirm these figures.

The peritoneal cavity is not a significant migratory route, less than 2% of total larvae produced during a natural infection can be recovered here and only 2% of viable NBL injected i.p. matured to muscle larvae. Since from 40-60% of NBL mature successfully after their intravenous injection (45,47) the low level of maturation indicates that NBL are unable to escape the peritoneal cavity. In fact, later in the infection, we observed a degree of larval growth in peritoneal larvae that suggested partial but fruitless development of free larvae in this site. These experiments are in close agreement with those of Dennis *et al*. (57) and suggest that less than 0.5% of muscle larvae burden is due to NBL that have migrated from the peritoneal cavity.

Attempts were also made to estimate the time taken by NBL to penetrate draining lymphatics or capillaries in the intestine after their birth. These experiments were based on the injection of adult worms intraluminally so that an unspecified time must be allowed for adult worm penetration into their intestinal niche. Under these conditions, NBL first appeared in lymph 50.3 ± 8.5 minutes after injection of adult worms. It is likely that less than half of this period was actually taken by NBL to migrate away from the intestinal mucosa(47,58). Many questions remain to be answered vis-à-vis the mechanisms of NBL migration through the mucosal tissues in the intestine, NBL penetration of the blood vessels, lymphatics and the striated muscle cells.

3.3. Newborn larvae surface antigens

A growing body of evidence testifies to the immunogenicity and protection-inducing capacities of the cuticular antigens of *T. spiralis*. While the earliest work (60) failed to demonstrate antibodies to NBL using immunofluorescence assays, similar techniques later detected antibodies in mice that had been immunized iv with 5,000-50,000 NBL(61). In the latter study, antibody was present as early as 2 weeks after infection. Functional cuticular differences were recognized by Mackenzie *et al*. (9) who found that the surface of infective muscle larvae and adult worms activated complement but that NBL did not. A more recent experiment, however, showed that NBL can activate the complement system via both the classical and the alternative pathways(62), which indicates that they not only can stimulate antibody production but also contain substances that can directly activate C3. Heat-stable factors in the serum of infected animals produced eosinophil adherence to all three stages of the parasite, but the appearance and maximum titer of these factors differed for each stage. These results, and those of Kazura and Grove (8), were the first to suggest that NBL surface components were immunologically distinct from those of other stages.

Shortly after this biochemical studies of the cuticle of *T. spiralis* by Philipp *et al*. (63) and Parkhouse *et al*. (64) showed qualitative variations in protein composition between muscle larvae, adults and NBL. In addition, there were quantitative changes in protein expression from day 1 to day 6 in adult worms. Recent structural and biochemical studies revealed the lipid components of muscle larvae(65) and an immunogenic protease was also identified in muscle larvae(66). With new techniques available to isolate the nurse cells(68), more findings will be made. In NBL, four distinct proteins with approximate molecular weights of 64K, 58K, 30K and 28K were found(63,64). The cuticle of *T. spiralis* is thus a dynamic organ, which exhibits dramatic changes at the molecular level between different stages of the life cycle and more gradual modifications during development within one stage.

3.4. Characteristics of newborn larvae of different ages

Ortega-Pierres *et al*. (69,70) used monoclonal antibody against the 64K protein to stain NBL and found that this antigen was present on NBL up to 8 hr old. Older larvae (8-48 hr) were not stained although the 64K antigen was still present along with three additional proteins (58, 34 and 30K) when examined by a surface radiolabelling and immunoprecipitation assay. This result indicates that the NBL cuticle is also dynamic and that the 64K protein on older larvae is probably not exposed as it is on younger larvae. If this is the case, older NBL may not be susceptible to antibody against the 64K protein *in vivo*. Binaghi *et al*. (71) studied the effect of NBL age on infectivity *in vivo* and resistance to cytotoxicity *in vitro*. Their results showed a 3-5 fold increase in muscle larvae burdens in normal mice receiving 20-hr-old larvae iv compared with mice receiving 2-hr-old larvae. After incubation with immune serum and peritoneal cells *in vitro* for 24 hr, between 50-100% of 2-hr NBL were dead, while 20-hr larvae were still alive after incubation for 48 hr. Gansmuller *et al*. (72) demonstrated that 2-hr larvae attracted only eosinophils after a 3-hr incubation *in vitro* whereas the adherent cell population was mostly macrophages on 20-hr-old larvae. These results suggested that older larvae were generally more resistant in both the normal host environment as well as in the presence of immune factors *in vitro*. It is possible that the effects of immune

serum and cells can be explained on the basis of the degree of exposure of the 64K protein *in vivo*. The vulnerability of 2-hr larvae is curiously at odds with the fact that NBL are most exposed to the host during the first 1-2 hours after production. A more recent study showed that NBL were killed *in vitro* by eosinophils in the presence of immune serum regardless of larval age(73). We addressed this question by *in vivo* examining the recirculation of NBL of different ages. The reason was that a difference might be seen in the migration process if 2-hr larvae were used rather than 24-hr larvae. We could find no real difference in recirculation (58).

3.5. Summary

Although minor discrepancies exist there is general agreement that NBL production begins on day 5/6 post infection and peaks on day 7. This is the period when the maximum number of adult worms is present in the intestine irrespective of infectious dose, and when the fecundity of females is at its peak. After day 8 a rapid decline in fecundity occurs and this is reflected in a precipitous drop in circulating larval numbers in the blood and lymph. More than 75% of total NBL are released in the first 3 days of production (6, 7, 8). Quantitative *in vivo* studies of the NBL migration route have emphasized different components of migration. Some larvae enter the blood via the thoracic duct, but disagreement exists as to the quantitative significance of this route. The alternative is a direct invasion of the intestinal capillaries that was found to be the major route in the only study that has examined portal vein traffic. Migration through the peritoneal cavity is negligible. Once in the blood NBL traverse the two visceral capillary beds they encounter (liver and lung) within minutes and can return to the venous drainage by 8 minutes. This is an active process requiring metabolic effort by the larvae. The speed of this return suggests that a considerable portion of migration is, for some larvae, intravascular. Although about 40% of NBL would be delivered to striated muscle during one passage in arterial capillaries only about half are actually removed in each vascular cycle. In total only some 40% per hour are permanently removed from the circulation. Although speculative it seems possible that a significant number of NBL migrate into and back out of muscle tissue. The mechanisms by which larvae identify and remain in their chosen site are entirely unclear. The *T. spiralis* NBL /rat system appears to offer several possibilities for study of the interaction between circulating nematodes and their hosts. This has been a very poorly explored area particularly with respect to site selection and the dynamics of recirculation, much more work needs to be done to understand these processes.

Despite their mundane morphology the larvae undergo what appears to be a differentiation, if not a developmental process, which is independent of their interaction with the host. These modifications are evident in the cuticular proteins as NBL maintained *in vitro* age. It has been suggested by at least two groups that changes in susceptibility to immunity are associated with aging. Curiously, young larvae (<6 hrs old) were less able to develop to mature muscle larvae than older larvae. In contrast to these findings we were unable to find differences in recirculation capacity, or development to mature muscle larvae when comparing larvae of 2 or 24 hours of age. Methodological differences may account for these discrepancies. While the observation that cuticular changes occur during the first 6 hours of independent larval life is interesting and needs to be pursued, there are no indications yet of what purpose or function these changes can have for the larvae. Indeed, it is difficult to relate cuticular molecular variations to larval resistance to host immunity since 50% of larvae disappear from the circulation in a primary infection 2 hours after their production, yet these larvae are the ones that appear most susceptible to immunity; older larvae being conspicuously resistant. Nevertheless, the age-related molecular heterogeneity of the cuticle may well provide important clues to the penetration of muscle and/or subsequent development of the young NBL.

4. HOST IMMUNITY TO NEWBORN LARVAE

4.1. The existence of host immunity *in vivo*

The earliest evidence for anti-NBL immunity was provided by Despommier (74) who found a 95% reduction in muscle larvae burden of rats immunized iv with NBL and then challenged with muscle larvae per os. Despite the strong immunity obtained, the author felt that NBL were not responsible as they did not possess beta-1 granules in their stichocytes. In the above experiments immunity was probably largely related to an anti-muscle larvae response that can lead to adult worm rejection and reduced fecundity in the challenge infection (10). James and Denham (17) provided a more definitive answer to this question. In their experiments, NBL were injected iv into mice which were then challenged with muscle larvae orally or with NBL iv. As assessed by muscle larvae burden, immunity was 100% effective against a NBL challenge but only 63% effective against a muscle larvae challenge. Similar observations were made by Ruitenberg and Steerenberg (75) and James *et al*.(7). The immunization procedure used by all of these groups was questioned by Perrudet-Badoux and Binaghi (38) on the basis that fetal calf serum used in the medium for larvae preparation could adhere to the cuticle and render the larvae immunogenic. To circumvent this Perrudet-Badoux and Binaghi (38) used an oral infection with muscle larvae to immunize mice and found complete protection against a NBL challenge. The question of the passive role of serum proteins therefore seems resolved. More recent studies by us demonstrated that inbred mice immunized with NBL showed wide variations in protection against challenge

infection (0-98%). Resistance to a challenge infection was influenced by genes that were not clearly related to the major histocompatibility complex (MHC) (45).

Immunity to NBL exists *in vivo* and can be extremely effective in preventing NBL from establishing in striated muscle. Immunization is effective with either an intravenous injection of NBL or an oral infection with muscle larvae. In all the above investigations, protection was gauged by muscle larvae burden. This information has set the stage for studies of where and when immunity occurs, particularly in the primary infection, and which mechanisms are effective *in vivo*. Since NBL begin their systemic migration immediately after their production in the intestine, they are likely to be susceptible to immunity anytime after they are born and any site through which they migrate. This is essentially the whole body. Indeed, our results showed that when we attempted to recover NBL from the peritoneal cavity, portal vein and thoracic duct lymph after challenge infection from previously immunized rats, the time of appearance of these larvae was significantly delayed and the recovery was significantly reduced in all sites examined(76). Obviously, the host immunity not only impeded NBL migration but also effectively destroyed them. Our later study also indicated that this immunity is specific to NBL, irrespective of their age(77). It should be noted that the experiments prior to ours did not exclude the possibility that the protection was also directed against maturing muscle larvae as they developed from the challenge NBL whereas our experiments were specifically designed to study immunity against NBL alone.

4.2. Stage specificity of host immunity against newborn larvae

Kazura and Grove(8)and, independently, MacKenzie *et al*.(9), first described an *in vitro* antibody-dependent cell-mediated destruction of NBL that was specific for this stage. The molecular basis for stage-specificity in the cuticular proteins of *T. spiralis* was later established by Philipp *et al.* (63,78) and Parkhouse *et al*.(64) as I have described above. Each study demonstrated that antibodies recognizing the surface antigens of one stage did not recognize those of other stages, although there might be low level of antigenic cross-reactivity with other nematodes (79,80).

4.3. Kinetics of the immune response to newborn larvae

Mackenzie *et al.* (9) studied the kinetics of anti-NBL immunity by examining the capacity of immune serum to mediate cell adherence *in vitro*. The activity was first found in serum collected 15 days after infection with 4,000 muscle larvae and the highest titer was observed 20 days after infection. Philipp *et al*.(78)and Jungery and Ogilvie (13), working with rats and mice respectively, found that the antibody-mediated cellular cytotoxic activity in serum was generated 15 days or 25 and 30 days after *T. spiralis* infection rats or NIH and C_3H mice respectively. Protection was demonstrated *in vivo* as in Wistar early as 10 days after a single iv injection of NBL in DBA/1 and AKR mice and this immunity was 96 to 98% protective against a NBL challenge(45). Because of the high degree of protection on day 10 it seems likely that NBL induced immunity was function prior to this. More recent data from us show that immunity can be demonstrated *in vivo* in rats 7 days after the initial infection and gains real strength by Day 10. This suggests that NBL are highly immunogenic and correlates the time of appearance of immunity with the precipitous decline in NBL output seen *in vivo* (76,77).

4.4. Summary

Immunity to NBL has slowly gained recognition since 1974 when James and Denham published their first study(17). Methodological criticisms of this and similar studies no longer appear valid and data now appearing indicate that the larvae are highly immunogenic and elicit a prompt and strong host response in most rodent strains examined. Immunity is detectable by day 7 in rats and achieves prominence around day 9 or 10 post infection when circulating NBL numbers undergo a precipitous decline. While other forms of immunity contribute to this decline in circulating larvae (notably anti-fecundity responses) it is very likely that anti-NBL immunity also plays its part. The temporal coincidence of these two forms of immunity, which appear to be quite strong in most rodent strains, suggests that the first 3 days of NBL production provide the great bulk of the muscle larvae burden of rodents.

5. MECHANISMS OF HOST IMMUNE RESPONSE TO NEWBORN LARVAE

5.1. Nonspecific resistance to newborn larvae

Grove and Civil (81) administrated Baccillus Calmette-Guerin (BCG, i.e., attenuated Mycobacterium bovis) iv to mice and then challenged them with NBL. The muscle larvae burden of these mice was examined 4 weeks after injection of NBL and showed a 25% reduction compared with controls. Since they found granulomatous inflammatory reactions in the lungs of these mice but not in the muscles invaded by the larvae, they suggested that the major site of NBL impairment was in the lungs. It has been suggested that BCG exerts its effect by increasing nonspecific resistance of the host to many parasitic infections, such as *Babesia*, *Plasmodium* (82), *Leishmania* species(83) and *Trypanosoma cruzi*(84). A similar reduction in muscle larvae burden was observed in rats treated with the adjuvant, *Corynebacterium parvum*, then infected with *T. spiralis*(85). The precise mechanism by which BCG enhances protection has not been investigated. Since it is known that BCG induces hyperplasia of mononuclear cells(86) and activates macrophages(87), these effects

may account for the increased protection described.

Bell *et al*. (45) provided evidence for nonspecific resistance to NBL *in vivo*. In these experiments, muscle larvae burdens of various inbred mice were examined after a single iv injection of NBL. A consistent pattern was observed in which C_3H strain mice always harbored the highest and DBA/1 mice the lowest burden of muscle larvae. Since these studies examined a single intravenous encounter with NBL in non immune mice, and the great majority of larvae have left the circulation by 24 hours(58), it is likely that the low muscle larvae burden of DBA/1 mice reflects a nonimmunological resistance to NBL. A comparison of muscle larvae burden with the MHC haplotype showed that k-haplotype mice(C_3H and AKR) had high muscle larvae burdens whereas q-haplotype mice(B10Q and DBA/1) had low burdens. This correlation suggests that components of nonspecific resistance are influenced by the MHC. These results parallel the earlier findings of Wassom *et al*. (43,44) in MHC association with high (H-2k) and low (H-2q) muscle larvae burdens after a single oral infection with muscle larvae. When muscle larvae burdens after a single injection of NBL were compared with those after a challenge injection with NBL there was a dissociation of "non-specific resistance" and immunity to the challenge. This dissociation of immunity to a challenge infection compared with resistance to a primary infection was interpreted as supporting the concept of a nonspecific resistance that was distinct from acquired immunity.

5.2. Cell-mediated immune response to newborn larvae

Moloney and Denham(18) studied cell-mediated immunity to NBL and found a 66% or 42% reduction of muscle larvae burden after NBL challenge of mice that had received spleen cells from donor mice hyperimmunized with NBL or muscle larvae, respectively. In the experiments, cells were passively transferred 24 hr prior to challenge so it is possible that B lymphocytes secreting antibodies led to an effective antibody-dependent cell-mediated cytotoxic reaction. Recently, a biophysical and biochemical study showed that ultraviolet light effectively impaired the T cell response to T. spiralis, leading to increased muscle larvae burden after infection(88). Whether this treatment affected the immunity to NBL and/or muscle larvae was not identified. Both studies did not characterize the cell population with regard to effector cell type and question of cell mediated immunity thus remains open.

5.3. Humoral immunity to newborn larvae

In the same experiments, Moloney and Denham(18)demonstrated that immune serum obtained from mice hyperimmunized with muscle larvae transferred 99.7% protection against a NBL challenge in recipient mice. The infectivity of NBL *in vivo* was reduced after incubation in immune serum *in vitro* and the effect of immune serum was lost after absorption with NBL. Immune serum always gave very strong protection if transferred before challenge but not when transferred 2 hr after challenge. Since *in vitro* incubation of NBL with immune serum alone neither alters the motility nor reduces the number of NBL(19), it is likely that immune serum alone may not be enough to reduce NBL infectivity *in vivo*. Other factors or effectors are likely to be involved and these factors in the nonimmune recipient presumably cooperate with specific antibodies acquired during *in vitro* incubation to influence NBL infectivity after their transfer *in vivo*. Our experiments also indicate that transferring immune thoracic duct lymphocytes failed to transfer protective immunity yet transferring immune serum did. Further fractionation of immune serum and immunofluorescence assay indicate that it is the IgG and possibly IgE that transferred protection(89).

5.4. Antibody-dependent cell-mediated immune response to newborn larva

Antibody-dependent cell-mediated cytotoxicity is a major host defense mechanism against infections with numerous parasites: Trypanosomes(90), trematodes (91,92), intestinal nematodes(93-95), and filarial worms (96-101). It is not surprising that *Trichinella spiralis* also fits this pattern at least with respect to the NBL(91,102). During *T. spiralis* infection a T-cell dependent peripheral blood eosinophilia occurs (103-105) which correlates with the level of plasma major basic protein (106). Eosinophils accumulate in the small intestinal mucosa (107) and increased numbers of eosinophils are found in the bone marrow during the NBL production period(108). Bartelmez *et al*.(109) showed that antigens of *T. spiralis* NBL as well as other stages stimulated mouse spleen cells to release eosinophil growth factor into the cultural medium *in vitro*. Similar response against muscle larvae indicates that it is a localized event(110). Other cell populations such as basophils (106)and peritoneal macrophages (111) are also increased. The granulopoietic stimulus provided by *T. spiralis* infections is therefore well established.

A considerable body of evidence implicates eosinophils and/or neutrophils in NBL killing. The first suggestion of a functional association between eosinophilia and protection was provided by Grove *et al*. (112) who showed that anti-eosinophil antisera administered *in vivo* increased the number of muscle larvae in treated rats but did not affect adult worms. The implied effect on NBL of eosinophils was quickly confirmed *in vitro* (8,9). Eosinophils appear to require antibody of the IgG class (19) to mediate cellular adherence but at least two processes lead to killing *in vitro*. The most extensively studied are the products of oxidative metabolism H_20_2 (peroxide) plus superoxide (0_2^-) and free Cl^- (113,114) which produce up to 100% killing *in vitro*. The observation that leukocytes from a

patient with chronic granulomatous disease (CGD) demonstrated significantly reduced larvicidal activity further suggests that the killing mechanism encompasses an oxidative response by normal leukocytes as granulocytes from CGD patients do not generate hydrogen peroxide (113). In addition, eosinophil major basic protein has also been shown to damage and kill NBL *in vitro*(115). Cells other than eosinophils also have a role, Mackenzie et al.(114) showed potent killing by neutrophils and Buys *et al*. (117) found that myeloperoxidase from neutrophils was more effective than eosinophil peroxidase in killing NBL. A variety of cell types can destroy NBL in addition to neutrophils and eosinophils. Mackenzie *et al*.(102) showed that mast cells, eosinophils, neutrophils and macrophages were all able to adhere to and destroy NBL during *in vitro* incubation even though significantly different adherence patterns were obtained. Cells do not attach to dead larvae leading to the suggestion that a chemotactic factor may be released from the live parasite which causes cellular adherence(100). Recent evidence shows that intestinal lamina propria cells are enriched with larval killing eosinophils(118), which confirms our finding that NBL are destroyed in significant quantity within the intestinal mucosa.

Evidence for antibody-dependent cell-mediated destruction of NBL is largely based on *in vitro* studies. In all these experiments it appeared not to matter whether leukocytes, especially eosinophils, were collected from normal hosts (19,72,102,113,116), from animals infected with *Schisotosoma mansoni* (117), *Mesocestoides* corti (13,69), or from *T. spiralis* infected mice(8), rats(120), and from *Trichinella* infected humans(73,121), as long as immune serum was present in the culture in addition to the cells NBL were killed. The results of Bruschi *et al*. also demonstrated that the sera factors inhibited neutrophil's oxidative metabolism, hence suggesting certain cytotoxic mechanisms other than production of superoxide anions may play a role(121).

The role of IgG has been unequivocally, established in a report describing a monoclonal IgG antibody against the 64K antigen of NBL which mediated eosinophil-dependent destruction of this stage *in vitro*. NBL treated with this monoclonal antibody *in vitro* and then injected into recipient mice that also received a further passive transfer of the anti-64K mAb for 3 days, showed a 36-51% reduction in muscle larvae burden. Conflicting evidence exists over the role of complement. Kazura and Grove(8)could find no effect but Mackenzie *et al*.(9)showed an effect of complement and so has a most recent study (62). *In vivo* studies have been limited, but in addition to the work of Grove *et al*. (112), described above, Dessein *et al*.(122) provided evidence in favor of a role for eosinophils and IgE. These authors administered rabbit anti-E chain antibodies to rats to suppress the production of IgE antibody. They then found that after infection with *T. spiralis*, the rats had reduced mast cell degranulation, decreased eosinophil numbers in the blood and tissues and increased muscle larvae burdens after a complete infection. Administration of IL-3 enhanced the IL-4-dependent IgE response whereas treatment with anti-IL-4 antibody suppressed such response(123,124), leaving no doubt that T cells are involved in the specific immune response. Our own data also have shown that larval migration is blocked irrespective of the site that larvae are introduced into the body. Larvae are promptly killed after intravenous or intraperitoneal injection into rats 15 or 16 days after a primary infection. In addition, if adult worms collected from non-immune rats are transplanted into the duodenum, more than 90% of the NBL produced do not enter draining lymphatics or capillaries (76,125), which is indicative of potent intestinal immunity.

Our electron microscopic studies revealed that in all cases larvae recovered from these local sites are covered with dense clusters of granulocytic cells(125). It appears that immunity, once induced, acts powerfully at any site that larvae are likely to contact in their systemic migration. Apparently the strength of this response is so great that few larvae will escape the confines of the local intestinal environment where they are produced. A most recent study in humans also confirmed this antibody-dependent cell-mediated cytotoxic effect on NBL(121). Obviously, what were discovered in rodents reflected what can take place in humans.

5.5. Summary

In vivo mechanisms and targets of immunity against NBL are the least understood component of this host parasite interaction. Considering the numerous *in vitro* descriptions of functional systems it is fair to conclude that NBL may be the targets of a diverse array of host effector processes. Both IgG and IgE antibodies have been implicated directly, while a variety of granulomatous or phagocytic cell types are able to mediate larval killing. Mechanisms of killing currently include oxidative processes and eosinophil major basic protein while a role for complement is also suggested by some studies. When all of these processes have been more precisely measured *in vivo* it seems likely that the NBL may be the target of more independent immunological and non immunological killing mechanisms than any other stage of the *T. spiralis* life cycle. Indeed *T. spiralis* may well be unique in the speed with which each life cycle stage induces immunity and the speed and degree to which the parasite modifies functional antigens during development and differentiation. If these processes are related then it appears that *T. spiralis* has specialized in a foreshortened life-cycle that remained strongly immunogenic thus outrunning immunity. Immunosuppression, although present(121,126,127), appears to be a secondary and less important process in the overall economy of the infectious event, especially

regarding the primary infection.

6. PERSPECTIVE

Anti-NBL immunity is a functional protective response in rodents and probably in swine during a primary infection. Evidence for this is derived from *in vitro* experiments and indirect and direct *in vivo* experimentation. The critical element in this assessment is the timing of immunity relative to NBL production. Immunity is observed within 3-4 days of initial larval release and coincides with a dramatic decline in the numbers of circulating NBL. Larvae are highly immunogenic in rats and in several mouse strains. As we are entering the molecular age in this particular field(128) and more techniques are developed in studying this parasite(129,130), I believe that there is still tremendous need to explore some of the fundamental biological properties of the NBL and the mechanisms of host immunity. The rat and newborn *T. spiralis* larvae model, thus, offers a useful and underexplored experimental model of the interaction of systemic nematode larvae and the host immune system.

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