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Time-course Changes in Antibody Responses to Different Stages of *Angiostrongylus cantonensis* in Experimentally Infected Rats

Chi Chang[1] and David Chao[2,*]

[1]*Department of Nursing, Mei-ho College, Pingtung, Taiwan 900, R.O.C.*
[2]*Department of Biology, National Sun Yat-sen University, Kaohsiung, Taiwan 804, R.O.C.*

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Chi Chang and David Chao (1996) TIme-course changes in antibody responses to different stages of *Angiostrongylus cantonensis* in experimentally infected rats. *Zoological Studies* **35**(1): 62-67. Sprague-Dawley rats were infected by stomach intubation of 200 third-stage larvae of *Angiostrongylus cantonensis*. Sera were collected at 2, 4, 6 and 8 weeks after infection. Sera from uninfected rats were used as the control. Humoral immune responses to different antigens were monitored by enzyme-linked immunosorbent assay (ELISA) and by crossed immunoelectrophoresis (CIE). Soluble antigens prepared from 3rd- and 5th-stage larvae and adult worms by homogenization and sonication were adjusted to the same protein concentrations and employed in ELISA and CIE. No antibodies against the 3 stages of the parasite were detected in the control sera or in sera collected from rats in the 2nd week of infection. Antibodies specific for 3rd-stage larvae appeared in the blood of the infected rats during the 4th week of infection as detected by ELISA but not by CIE. Peak ELISA levels against 5th-stage larvae and adults were observed in the 8th week of infection. Sera collected in the 4th and 8th week of infection contained detectable antibodies against 5th-stage larvae by CIE. Antigens extracted from 5th-stage larvae developed 8 and 4 peaks with sera collected in the 4th and 8th week, respectively, while those from adults formed 7 and 5 peaks, respectively.

Key words: *Angiostrongylus cantonensis*, Experimental infection, ELISA, CIE, Stage-specificity.

Angiostrongylus cantonensis, the nematode parasite which inhabits in the pulmonary arteries and heart of rodents, has been recognized as the primary causative agent of eosinophilic meningoencephalitis in Taiwan since 1944 (Nomura and Lin 1945). The disease is now considered to be one of the most important parasitic zoonoses in Southeast Asia and the Pacific area (Bhaibulaya 1979, Chen 1979, Ko et al. 1984).

In the investigations of the immune responses to *A. cantonensis*, analyses of antigens from mammalian parasitic stages have become essential steps toward understanding and manipulating the interaction between this parasite and its host. In the rat host, *A. cantonensis* develops from 3rd-stage larvae into adults through various stages during its life cycle and may live for over 1 year (Yong and Dobson 1982a). Immune responses directed against these stages are important in the control of the parasite during infections (Yong and Dobson 1982b,c,d 1983, Wang et al. 1989). However, investigations have been hindered by such difficulties as collecting sufficient amounts of 3rd-stage larvae. The purpose of the present study is to provide some information of humoral immune responses in experimentally infected rats during the course of early infection with antigens prepared from different developmental stages of *A. cantonensis*.

MATERIALS AND METHODS

Parasites

The Taiwan strain of *A. cantonensis* used in this study was originally isolated from the giant African snail, *Achatina fulica*, collected from Neihu,

*To whom all correspondence and reprint requests should be addressed.

Taipei in 1980. It was maintained in this laboratory by cycling through the planorbid snail, *Biomphalaria glabrata*, and Sprague-Dawley rats. The 1st-stage larvae of *A. cantonensis* were recovered from infected rat feces and fed to snails (Bhaibulaya 1975). Third-stage larvae were obtained from the tissue of infected snails which had been artificially digested with 0.6% pepsin-HCl (pH 2.5) for 1 h. Rats were infected by stomach intubation.

Animals

Female 10-week-old Sprague-Dawley (SD) strain rats were purchased from the Animal Center, National Yang-Ming Medical College, Taipei. They were housed in plastic cages and given food and water ad libitum.

Antigens

Soluble antigen extracts were prepared from homogenized 3rd- and 5th-stage larvae and adult worms. Third-stage larvae were obtained from infected snails. Fifth-stage larvae were obtained from the brains of infected rats 17 d after infection. Adult worms were collected from the pulmonary arteries and hearts of other infected rats. The worms were washed 3 times with normal saline and 3 times with phosphate-buffered saline (PBS, 0.2 M, pH 7.2). They were homogenized in a ground glass tissue grinder which contained a small volume of PBS. The homogenized mixture was sonicated in an ultrasonic disintegrator (Soniprep 150, MSE Scientific Instruments, Manor Royal, U.K.). The sonicate was centrifuged at 12 500 g for 45 min. Aliquots of supernatant extracts were stored at −70 °C. All extraction steps were conducted on ice. Protein concentrations of the extracts were determined by the Lowry method (Lowry et al. 1951). These extracts were adjusted to the same protein concentrations and employed in the tests.

Antisera

Rats were infected with 200 active 3rd-stage larvae by stomach intubation. After infection, they were bled by cardiac puncture at 2-week intervals. Sera were collected from blood by centrifugation at 2 000 g for 20 min and stored at −20 °C until used. Sera collected from uninfected rats were employed as controls.

Enzyme-linked immunosorbent assay (ELISA)

The procedure followed for ELISA was modified from the indirect micro-ELISA test described by Voller et al. (1976). Briefly, 20 μl of antigen extract solution containing 10 μg/ml protein diluted in pH 9.6 carbonate buffer (0.06 M) was used to sensitize the wells of micro-ELISA plates. Serum samples (1/20 dilution) were prepared in duplicate to test against equal protein concentrations of each antigen extract preparation absorbed to the wells. Goat anti-rat IgG antibody labelled with alkaline phosphatase (1/800 dilution, Kirkegaard & Perry Laboratories) was used to measure humoral antibodies against different stages of the parasite in the infected rats. The enzyme reaction was monitored 30 min after the addition of substrate solution (paranitrophenyl phosphate in pH 9.8 diethanolamine buffer) by photometric measurements of optical density at 405 nm (Titertek Multiskan, Flow Laboratories).

Crossed immunoelectrophoresis (CIE)

CIE was conducted using the method described by Weeke (1973). A 15-ml suspension of 1% agarose solubilized in heated pH 8.6 tris-diethylbarbituric acid buffer (8.86 g/l tris, 4.48 g/l diethylbarbituric acid, 0.1 g/l calcium lactate, and 0.20 g/l sodium azide) was poured onto the hydrophilic surface of a 100-mm strip of Gel Bond film (Marine Colloids Division, FMC Co. Bioproducts, Rockland, Maine). After solidifying, a 10-μl sample of extract was placed in a 4-mm diameter well. Electrophoresis of the first dimension was conducted at 10 V/cm for 90 min at 15 °C in a Bio-Phoresis horizontal cell (Bio-Rad Laboratories, Rockville, New York). After the electrophoretic separation was completed, a portion of the gel was removed, leaving a strip containing the fractionated extract. An 11-ml sample of agarose containing 1.0 ml of the appropriate serum was poured onto the area of the Gel Bond from which the agarose was removed. Electrophoresis in the second direction was conducted at 2 V/cm for 18 h at 15 °C. After electrophoresis, the gels were pressed, washed 3 times, and stained with Coomassie Brilliant Blue R-250.

RESULTS

No significant increase of antibodies against any of the 3 stages could be detected by ELISA during the first 2 weeks of infection (Fig. 1). Third-stage larva-specific antibodies appeared in the

blood of infected rats during the 4th week of infection. Antibody levels were detected in rat sera in the 4th week of infection. The highest ELISA value against 5-stage larvae was found during the 8th week of infection. Similar antibody levels were demonstrated with extracts of adult worms.

Extracts of each of the stages formed no precipitin lines with control serum in CIE. Precipitin lines specific to no stage of the parasite could be detected by CIE during the first 2 weeks of infection.

The antigenic differences among different stages could be detected by CIE with the antisera collected at either the 4th or the 8th week of infection (Figs. 2,3). More common antigens were found between the 5th-stage larvae and adults than between the other stages. Antisera collected at 4 and 8 weeks post-infection developed 8 and 4 peaks, respectively, when reacted with antigens derived from 5th-stage larvae. Antigens of adults formed 7 and 5 peaks, respectively, compared to 4 and 3 peaks, respectively, for 3rd-stage larvae with the antisera from rats infected for 4 and 8 weeks. Antibodies in the rat sera 4 weeks post-infection reacted more strongly with antigens in extracts of 5th-stage larvae than those of adults. At least 2 unique precipitin peaks with unfused bases could be considered as stage-specific antigen-antibody systems detected by CIE (Fig. 2C, arrows).

DISCUSSION

In addition to being the infective form of *A. cantonensis*, the 3rd-stage larvae are also the migrating form; migrating from the intestines through the circulatory system to the meninges, the spinal cord, and the brain. However, they remain as 3rd-stage larvae for only a short period. Within 4 to 6 days, they complete migration and undergo 1 additional molt in brain. About 1 week later, they develop into 5th-stage larvae and leave the brain. The appearance of antibodies to the antigens of each stage were roughly correlated with this life cycle. Resistance which developed after exposure to the full antigenic component of infective larvae was comparatively strong. Previous work demonstated that rats immunized with live third-stage larvae were better protected than rats immunized with somatic antigens (Wang et al. 1989). Rats given 200 larvae retained fewer worms following a challenge infection when compared to rats given 50, 100, or 400 larvae. Rats in this study were experimentally infected with 200 active 3rd-stage larvae based on this finding.

It is generally agreed that *A. cantonensis* infections are complex and involve a balance between host immune responses and parasite virulence factors. The fates of hosts and parasites in permissive rat hosts or in non-permissive human hosts are totally different. Unlike infections in rats, worm-

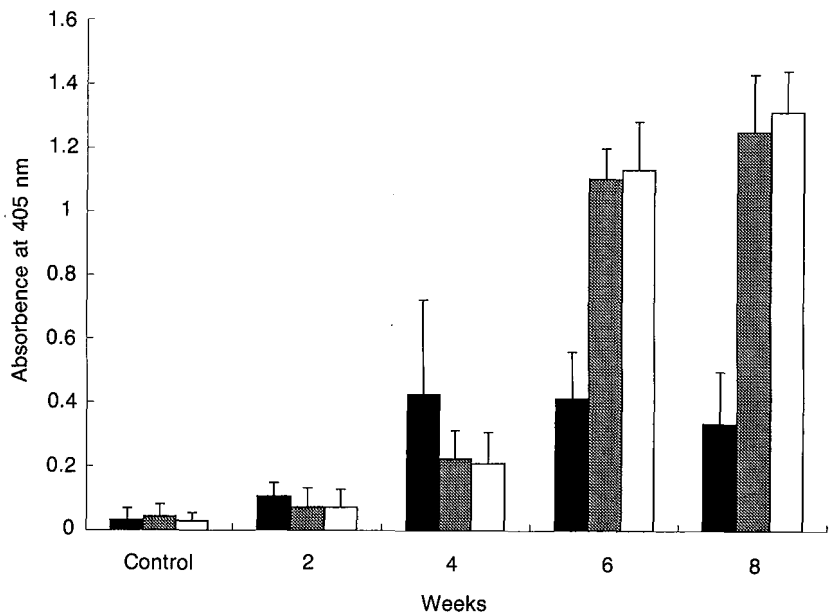

Fig. 1. Variation in antibody levels to stage-antigens of *A. cantonensis* in rats infected with 200 3rd-stage larvae as detected by ELISA. Solid bar, 3rd-stage larval antigen; shadowed bar, 5th-stage larval antigen; open bar, adult antigen. $N = 5$.

proven diagnosis in human angiostrongyliasis is very difficult, since the parasite usually remains in the central nervous system in human hosts in its 5th-stage larval form. Numerous studies have concerned immunodiagnosis of *A. cantonensis* infections in humans (Chen and Suzuki 1974, Welch et al. 1980). However, satisfactory diagnosis has been hampered by the lack of knowledge of the specific antigens presented by transient larval stages and the specificity or sensitivity of the immunodiagnostic techniques. Dharmkrong-at and Sirisinha (1983) found antigenic differences among different stages of *A. cantonensis*. The present work demonstrates that soluble antigens derived from different stages of *A. cantonensis* contain different antigenic components. This may partially explain why, in a previous study, different stages induced different levels of protection in rats (Wang et al. 1989). Many of the adult antigens described by this study may not be present in human infections. Fifth-stage larva-specific antibodies need to be further investigated for their usefulness in immunodiagnostic application.

This study focuses on the dynamics of each antigenic component of *A. cantonensis* during the life cycle and the stage-specific humoral responses in the course of the early infection. ELISA is a very sensitive method for the detection of specific antibodies to each stage of the parasite. It is also a quantitative immunochemical procedure. Yong and Dobson (1982c) showed that antibody titers reached a peak 50 days after infection, whereas in this study, rats infected for 8 weeks exhibited the highest ELISA values of early infection during the investigation period which correlates well with the results of the earlier study.

CIE characterizes the complexity of reactions of antisera. Different antibodies appear in the host sera at intervals following infection. Infections with *A. cantonensis* evoke the synthesis of antibodies which are elicited by different specific antigens of the differentiating parasites producing multi-

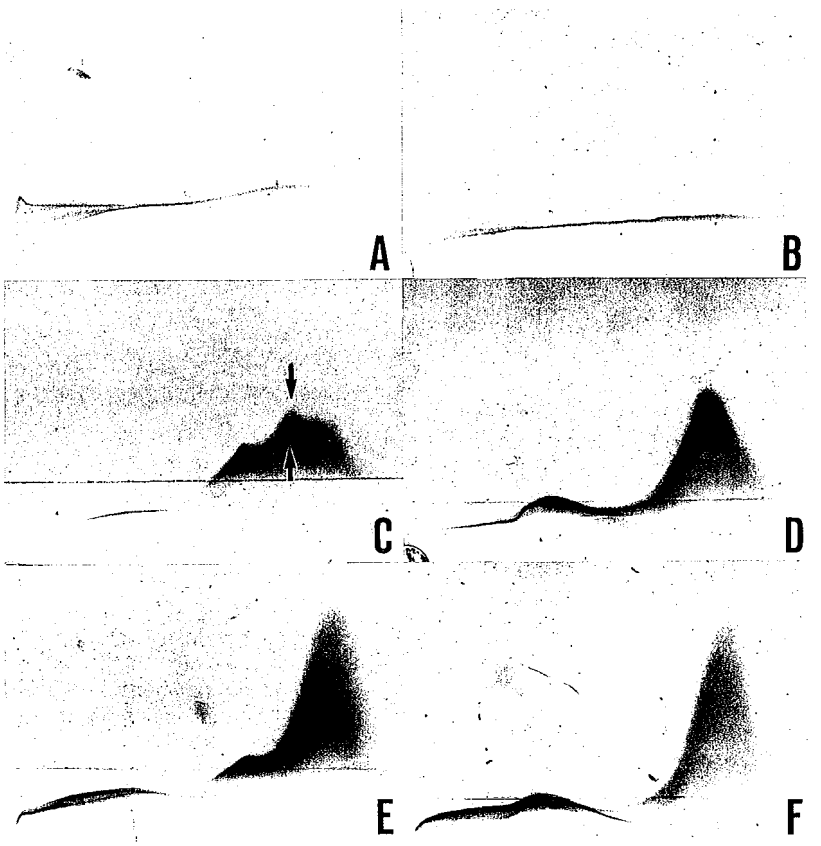

Fig. 2. CIE characterization of antisera against stage antigens of *A. cantonensis*. Precipitin reactions are shown for 4-week antisera reacted with 3rd-stage larval antigens (A), 8-week antisera reacted with 3rd-stage larval antigens (B), 4-week antisera reacted with 5th-stage larval antigens (C), 8-week antisera reacted with 5th-stage larval antigens (D), 4-week antisera reacted with adult antigens (E), and 8-week antisera reacted with adult antigens (F).

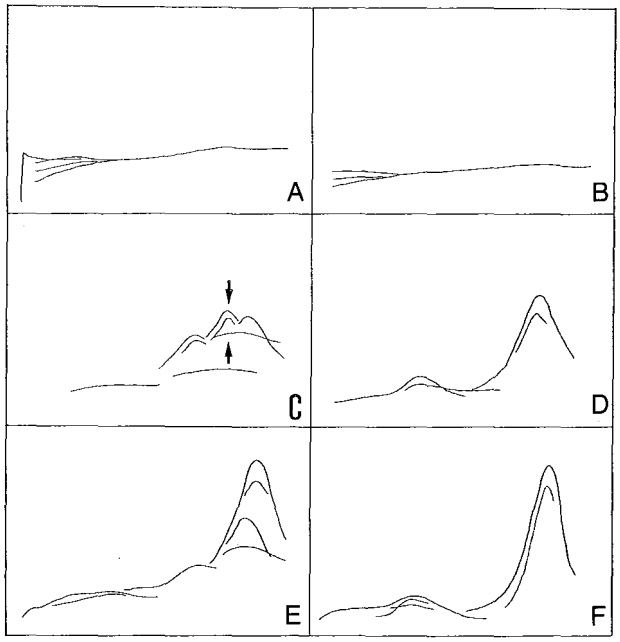

Fig. 3. Line drawings of the CIE precipitin peaks observed in Fig. 2.

specific antisera characteristic of each developmental stage. CIE also indicated that 3rd- and 5th-stage larvae differed in at least 4 to 6 antigens, while 5th-stage larvae and adults differed in at least 1 to 3 antigens. There are variations in the staining of individual precipitin peaks. Some appeared as sharp peaks while others were somewhat diffuse. Diffuse precipitates were counted as single precipitin peaks and considered to represent one antigen-antibody system. Differences in the time of appearance and amount of antibodies detected by different methods suggest that the antibodies in the serological tests may recognize different antigens. The antisera from rat infected with live 3rd-stage larvae do not respond to 5th-stage larva-specific antigens until 4 weeks after infection. Some of the 3rd-stage larva-specific antigens detected by CIE may stimulate antibodies responsible for resistance to infections. Antibody responses directed against 5th-stage larvae or adults appeared stronger than those against 3rd-stage larvae. The question of whether these responses are sufficient to induce protective immunity, or are even related to protection, remains to be tested and clarified. If similar antibodies can be found in clinically immune individuals, these antigens may be candidates for production of vaccine.

Immunity against infection by *A. cantonensis* in rodent hosts has been documented by several investigators. The excretory-secretory (ES) antigens prepared from adult worms (Uahkowithchai et al. 1977), live infective 3rd-stage larvae (Cross 1979, Yong and Dobson 1982b, Wang et al. 1989), irradiated 3rd-stage larvae (Lee 1969, Kamath et al. 1986), crude antigenic extract of 3rd-stage larvae (Dharmkrong-at and Sirisinha 1985, Kum and Ko 1987), 5th-stage larvae (Wang et al. 1989), and adult worms (Wang et al. 1989) have been employed to immunize animals against challenge infections. Rats immunized with live parasites showed fewer worms, but also showed low serum antibody values (Wang et al. 1989). The effects of antibodies and their eliciting antigens during the course of infection as well as other components of the immune system on resistance to *A. cantonensis* remain to be investigated and elucidated.

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大鼠實驗感染廣東住血線蟲對不同期蟲體抗原免疫反應之時程變化

張　琪[1]　趙大衛[2]

S-D品系大鼠以食道灌注法實驗感染200隻臺灣株的廣東住血線蟲第三期幼蟲(L_3)後，每隔二週收集抗血清至第八週，並以正常大鼠血清爲對照，使用酵素免疫分析法(ELISA)及交叉免疫電泳(CIE)來追蹤其對不同期蟲體之抗體反應。以組織研磨及超音波震碎法萃取不同時期廣東住血線蟲蟲體抗原，包括L_3、第五期幼蟲(L5)及成蟲(Ad)。在感染之最初二週內難以偵測到抗體反應；在感染的第四週大鼠體內第三期幼蟲已不存在時，用ELISA偵測到抗第三期的幼蟲之抗體達最高；同樣以ELISA分析，抗第五期幼蟲及成蟲的抗體在第八週時達最高。而以CIE分析第四及第八週的抗血清，分別有針對第五期幼蟲而形成的8及4個沉澱峰，而與成蟲抗原反應則分別出現7及5條的沉澱峰。

關鍵詞：廣東住血線蟲，實驗感染，酵素免疫分析法，交叉免疫電泳，蟲期專一性。

[1] 屏東美和護理管理專科學校護理科

[2] 國立中山大學生物學系