HISTOCHEMICAL ALTERATIONS OF INFECTIVE THIRD-STAGE HOOKWORM LARVAE (L_3) IN VACCINATED MICE

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Abstract. To study the histochemical alterations of hookworm L_3 administered in a challenge dose to mice vaccinated previously with the larvae. Male Kunming strain mice vaccinated subcutaneously with 500 living *Ancylostoma caninum* L_3 once every 2 weeks for a total of three immunizations before a final challenge with 500 L_3 one week after the final immunization. The abdominal skin with underlying subcutaneous tissue and muscle were removed from the site of percutaneous challenge entry (from 2-3 mice), and fixed in absolute alcohol, cold acetone and 10% neutralized formalin. The tissue sections containing the L_3 from the challenge dose were then stained histochemically of glycogen, RNA, DNA alkaline protein, acid mucopolysaccharide, collagen, reticulin, alkaline phosphatase (AKP) and adenosine triphosphatase (ATPase). Skin samples from non-immunized mice that were also subcutaneously inoculated with the L_3 served as negative control. The L_3 identified in cutaneous sections from vaccinated mice at 6-72 hours post-challenge exhibited reductions in parasite glycogen, alkaline protein, RNA and DNA, as well as reductions in acid mucopolysaccharide, collagen and reticulin contents in the parasite cuticle. There were also reduced enzyme AKP and ATPase activities. In contrast L_3 identified in sections from non-immunized mice exhibited a normal histochemical appearance, as did some L_3 who survived in vaccinated mice at 7-14 days post-challenge. Vaccination results in hookworm L_3 damage which is manifested by reduced histochemical staining for the challenge inoculum of parasites. There is also reduced hydrolytic enzyme activity. The observed changes could reflect either host-mediated parasite structural damage and disintegration or possibly anti-metabolic properties of the host immune response.

INTRODUCTION

Human hookworm infection is an important public health problem and a leading cause of anemia and malnutrition in China. In the early 1990s the extent of the hookworm problem in China became clear based on the fecal examinations of 1,477,742 individuals in every Chinese province. From this nationwide survey it was determined that 17% of the population is infected with hookworms, an estimated 194 million cases (Hotez *et al*, 1997). Over the last decade the prevalence and intensity of hookworm infection has decreased in some provinces where economic development has occurred at a rapid pace (Sun *et al*, 1999). However, in other regions, especially where poor conditions persist, hookworm has increased in prevalence and intensity (Liu *et al*, 1999). Because anthelminthic chemotherapy has failed to control hookworm in some hyperendemic areas, we have looked to the possibility of employing biotechnology to explore alternative control methods, including hookworm vaccine development (Hotez *et al*, 1996). As a first step was explored the mouse as a suitable host for hookworm immunity and showed that repeated dosing of third-stage infective hookworm larvae (L_3) will elicit resistance to challenge infections of the dog hookworm *Ancylostoma caninum* (Xiao *et al*, 1 998a; Ghosh *et al*, 1996). This confirms an earlier observation made by Kerr (1936). The resistance is not absolute, but instead is manifested as a reduction in the number of L_3 recovered from target organs including lungs, gastrointestinal tract and muscles (Xiao *et al*, 1998a). Murine immune responses after L_3 vaccination are compared of both humoral ant-L_3 antibody responses (Xue *et al*, 1999), as well as prominent cellular inflammation in the lungs, skin and abdominal musculature (Yang *et al* 1998). The cellular responses are comprised of eosinophils and mast cells suggestive of possible Th2 type responses (Xiao *et al*, 1998b; Yang *et al*, submitted); an observation also suggested by the prominent increases noted in total IgE after L_3 vaccination (Ghosh and Hotez, submitted). However, the prominent granulomata noted in response to vaccination indicates that Thl responses are also important for protection (Yang *et al*, 1998). he protection afforded by living L_3 vaccination is not long-lived (Xiao *et al* 1999), although it can be increased by first attenuating the

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larvae with ionizing radiation unpublished observation). Protection occurs either by vaccinating through the oral or subcutaneous routes. Here we further investigate the mechanism of protection by examining the cutaneous histochemical alteration of the parasite after subcutaneous vaccination and larval challenge.

MATERIALS AND METHODS

Murine hookworm L_3 vaccinations and challenge

A Shanghai strain of *A. caninum* hookworm was maintained in hybrid dogs; living L_3 were collected from coproculture as described previously (Xiao *et al*, 1998a). Thirty outbred male (Kunming strain) mice, each weighing 18-22 g, were immunized subcutaneously with 500 living *A. caninum* L_3 once every 2 weeks for three times as described previously (Yang *et al*, 1998). One week after the final immunization, the mice were challenged percutaneously with 500 L_3 larvae via the abdominal skin.

Histochemical staining of L_3 in cutaneous sections

At different intervals ranging from 6-72 hours and then 7-14 days post-L_3-challenge, the abdominal skin with underlying subcutaneous tissue and muscle at the site of percutaneous challenge entry was removed from groups of two to three mice. Each skin sample was cut into three pieces and fixed in absolute alcohol, cold acetone and 10% neutralized formalin. Skin from non-immunized mice infected percutaneously with 500 L_3 served as negative control. Each fixed skin sample was further cut into 3-5 small pieces and 50 tissue sections of 7-10μm thickness were prepared from each small piece. The tissue sections were then stained histochemically (Pearse, 1960), using-periodic acid-Schiff (PAS) in the presence and absence of amylase (glycogen), Mazia's acidophilic stain (alkaline protein), Feulgen-Schiff (DNA), Brachet methyl green-pyronin method (RNA), Mallory trichrome (Collagen), steedman's Alcian blue (acid mucopolysaccharide), Foot silver stain (reticulin), and two enzymatic reactions for alkaline phosphatase (AKP) using the Gomori's calcium-cobalt method and adenosine triphosphatase (ATPase) using the Padykula-Herman's calcium method. For each stain the tissue sections from immunized and non-immunized mice were put in the same jar. A total of 100-150 sections from each time group of immunized mice were examined and the intensity of histochemical reactions appeared in L_3 were visually compared with the corresponding group from sections of non-immunized mice.

RESULTS

Histochemical evidence for L_3 structural degradation and necrosis

Hookworm L_3 stain prominently with PAS as shown from the section harvested from a non-immunized mouse (Fig 1). However, L_3 from immunized mice showed evidence of parasite destruction, with a reduction in PAS staining. Many of these dead and dying larvae were contained within granulomata. As shown in Table 1, after 6-72, hours post-challenge, 26-42% and 14-64% of the larvae examined in sections of vaccinated mice exhibited either loss or disappearance in PAS staining (Figs 2, 3), respectively, suggestive of decreased glycogen content in their muscle. In contrast, there was no loss in L_3-associated PAS staining in the sections obtained from non-immunized mice. Similar loss of definition could be identified for other structural components from sections recovered from immunized mice compared to non-immunized mice, which contained intact, viable L_3. Greater losses of parasite RNA (Figs 4, 5), DNA (Figs 6, 7), alkaline protein (Figs 8, 9), collagen (Figs 10-12), reticulin (Figs 13, 14) and acid mucopolysaccharide (Figs 15, 16) were observed in the sections obtained from L_3-vaccinated compared to non-immunized mice. All of the sections examined from non-immunized mice contained normal appearing L_3 (Table 1). The percentages of damaged L_3 with reduced RNA and alkaline protein content are also summarized in Table 1. In most cases the percentages of normal appearing larva decreased with increasing time after challenge. However at extended periods beyond 72 hours after L_3 vaccination some challenge L_3 survive the host inflammatory response intact (Xiao *et al*, 1998a). We observed that 7-14 days post-challenge, those surviving larvae had normal glycogen, alkaline protein, RNA and DNA contents, as well as collagen, reticulin and acid mucopolysaccharide structures (data not shown).

Histochemical evidence for alterations in L_3 hydrolytic enzymes

The L_3 seen in sections from non-immunized mice exhibited strong reactivity for stains which appear as enzyme products of ATPase and AKP (Fig 17). However, after L_3-vaccinated mice were challenged with L_3 for 6-72 hours, the larvae in cutaneous and muscular sections showed significant losses of ATPase and AKP enzyme activity staining (Figs

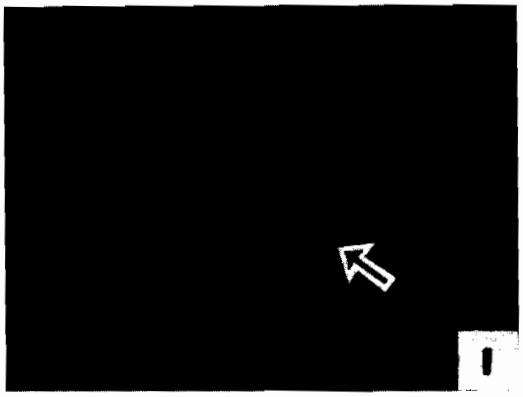

Fig 1–24 hours after infection in non-immunized mice, showing abundant glycogen content in muscle of L_3 (arrow) in dermis x 200 PAS method (Hotchkiss).

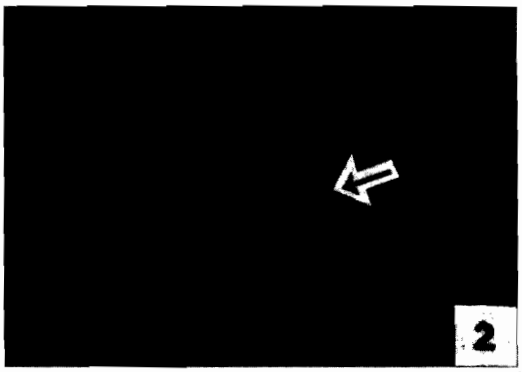

Fig 2–72 hours after challenge in immunized mice, showing reduction of glycogen content in muscle of larva (arrow) inside the granuloma in the hypodermis x 200 PAS method (Hotchkiss).

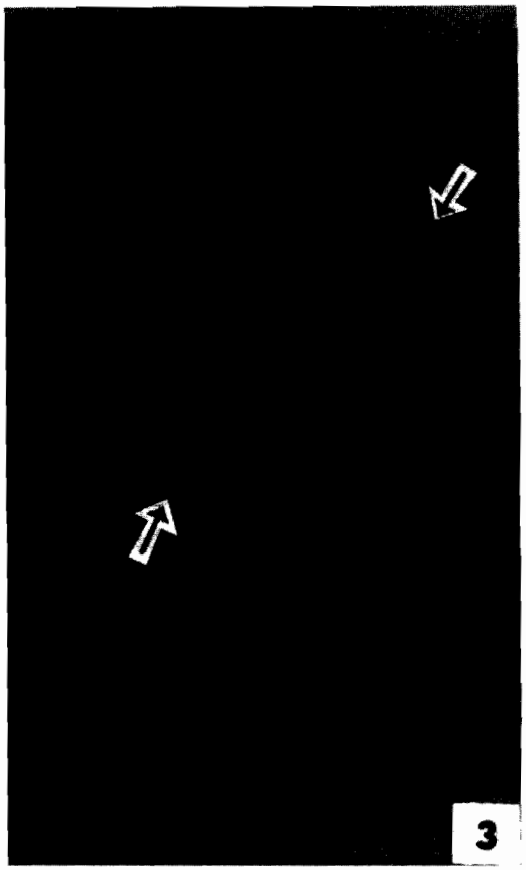

Fig 3–14 days after challenge in immunized mice, showing marked decrease or disappearance of glycogen in dead larvae (arrow) inside the granuloma in dermis and hypodermis x 100 PAS method (Hotchkiss).

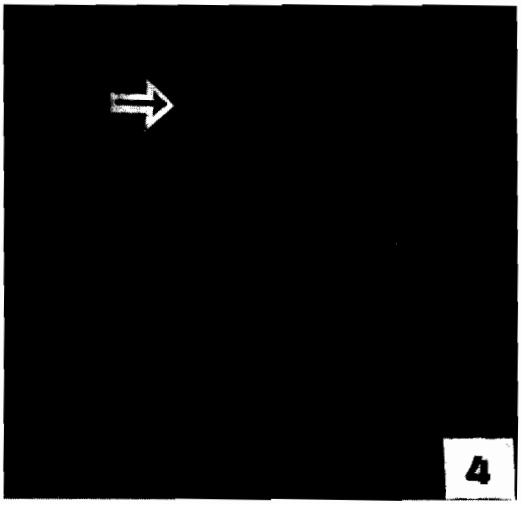

Fig 4–24 hours after infection in non-immunized mice, showing abundant RNA in the cuticle of larva (arrow) located in the dermis x 400 Brachet methyl green-pyronin method.

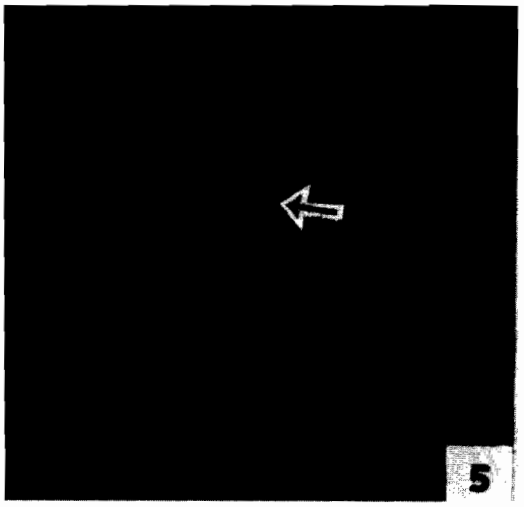

Fig 5–6 hours after challenge in immunized mice, showing marked decrease of RNA in the cuticle of a degenerated larva (arrow) located in dermis x 400 Brachet's methyl green-pyronin method.

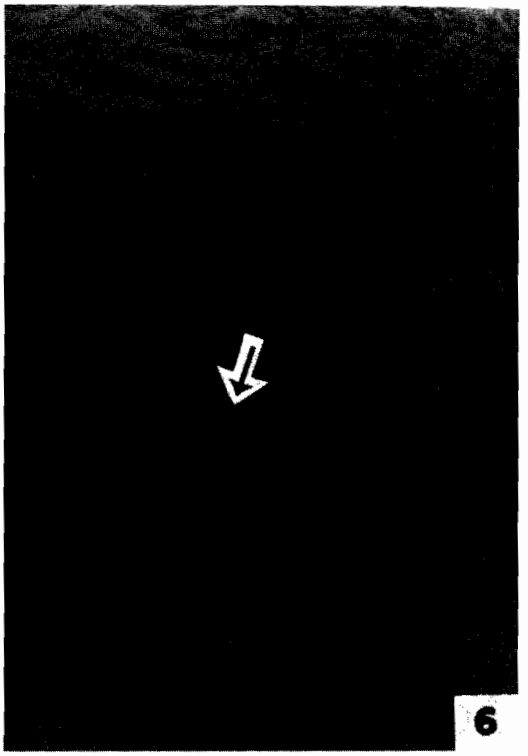

Fig 6–24 hours after infection in non-immunized mice, showing abundant DNA in the cell nucleuses of larva (arrow) located in dermis x 200 Feulgen-Schiff method.

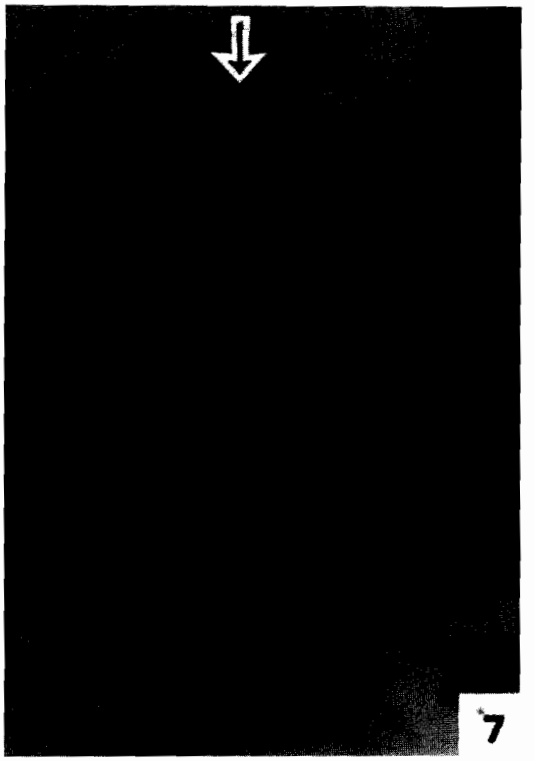

Fig 7–24 hours after challenge in immunized mice, showing decrease of DNA in cell nucleus of a degenerated larvae (arrow) inside the granuloma located in dermis x 400 Feulgen-Schiff method.

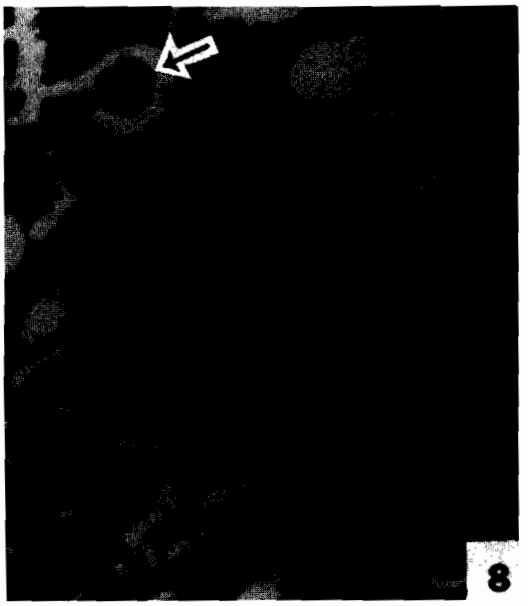

Fig 8–24 hours after infection in non-immunized mice, showing abundant alkaline protein in the cuticle of larva (arrow) x 400 Mazia's acidophilic stain.

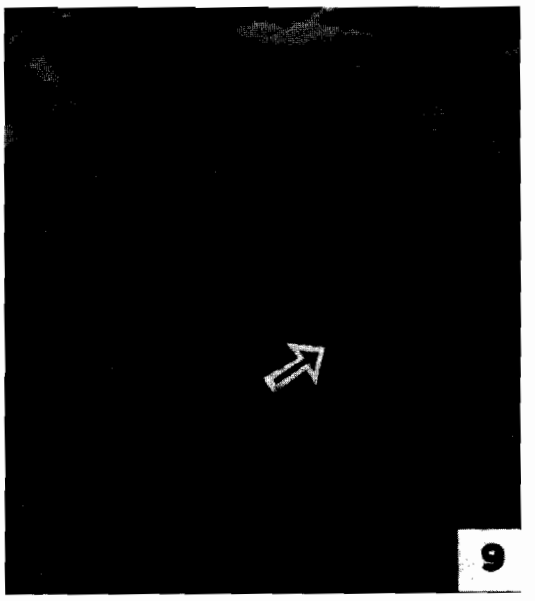

Fig 9–24 hours after challenge in immunized mice, showing vesiculation and disappearance of alkaline protein in the cuticle of larva (arrow) located in dermis x 400 Mazia'acidophilic stain

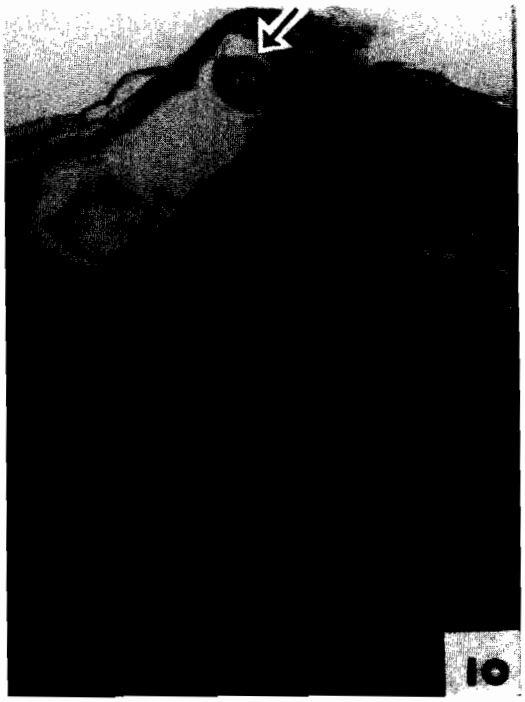

Fig 10–6 hours after infection in non-immunized mice, showing abundant collagen in the cuticle of larva (arrow) located in stratum spinosum of epidermis x 400 Mallory trichrome method.

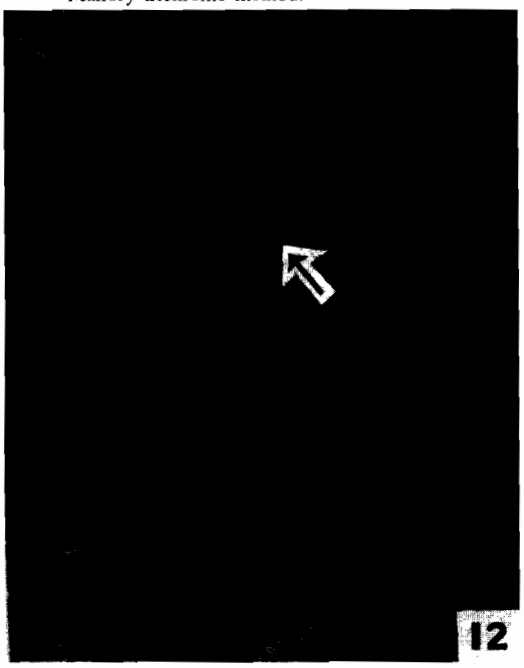

Fig 12–14 days after challenge in immunized mice, showing marked decrease or disappearance of collagen in the cuticle of larva (arrow) located in abdominal muscle and formation of many collagen around the larva x 200 Mallory trichrome method.

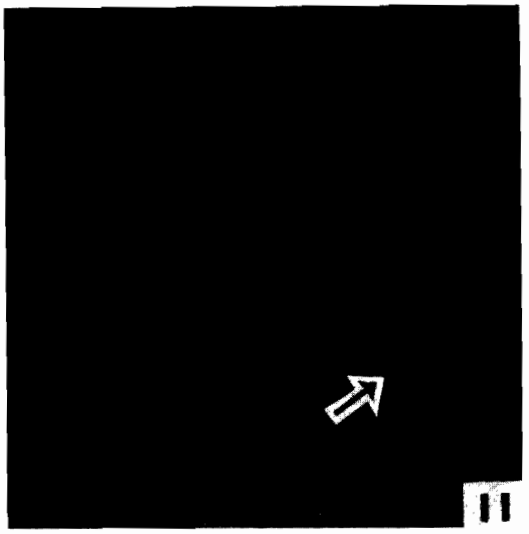

Fig 11–7 days after challenge in immunized mice, showing appearance of many collagenous fiber (arrow) around the degenerated larva located in abdominal muscle x 200 Mallory trichrome method.

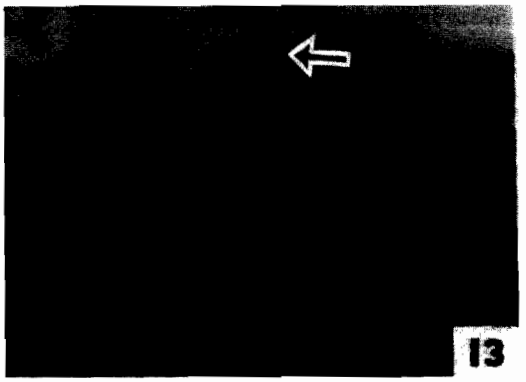

Fig 13–6 hours after infection in non-immunized mice, showing abundant reticulin in the cuticle (arrow) of larva located in dermis x 200 Foot silver stain.

Fig 14–24 hours after challenge in immunized mice, showing decrease of reticulin in the cuticle (arrow) of larva located in dermis x 200 Foot silver stain.

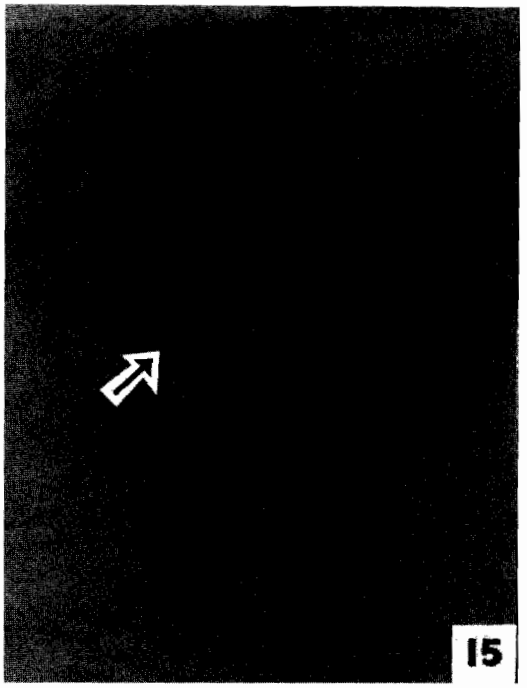

Fig 15–6 hours after infection in non-immunized mice, showing abundant acid mucopolysaccharide in the cuticle (arrow) of larva in dermis x 400 Steedman's Alcian blue method.

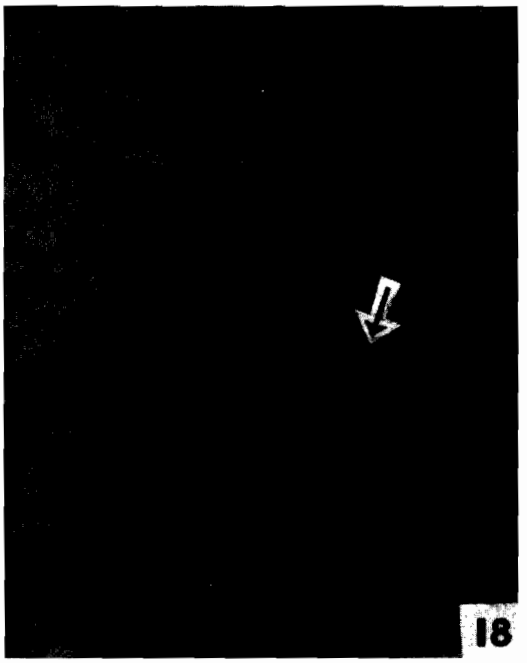

Fig 18–72 hours after challenge in immunized mice, showing decrease of ATPase activity in the cuticle (arrow) of larva located in hypodermis x 100 Padykula-Herman's calcium method.

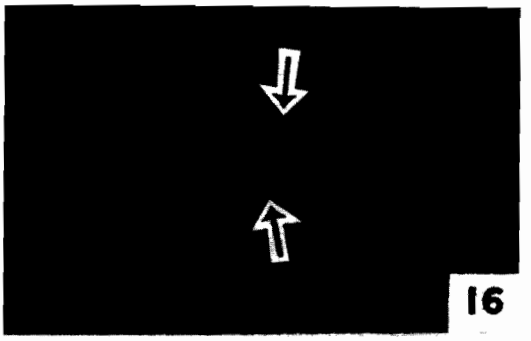

Fig 16–72 hours after challenge in immunized mice, showing disappearance of acid mucopolysaccharide in the cuticle (arrow) of larva inside a granuloma and emergence of many acid mucopolysaccharide around the larva x 200 Steedman's Alcian blue stain.

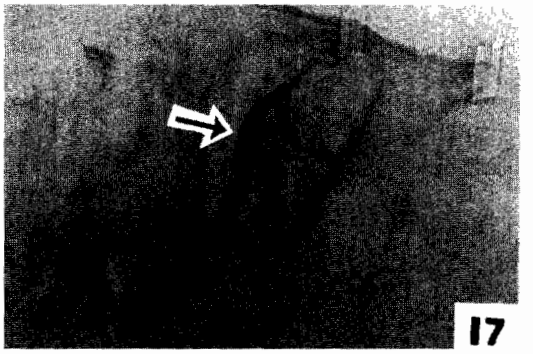

Fig 17–24 hours after infection in non-immunized mice, showing strong activity of alkaline phosphatase (AKP) in the cuticle (arrow) of the larva in dermis x 200 Gomori's calcium-cobalt method.

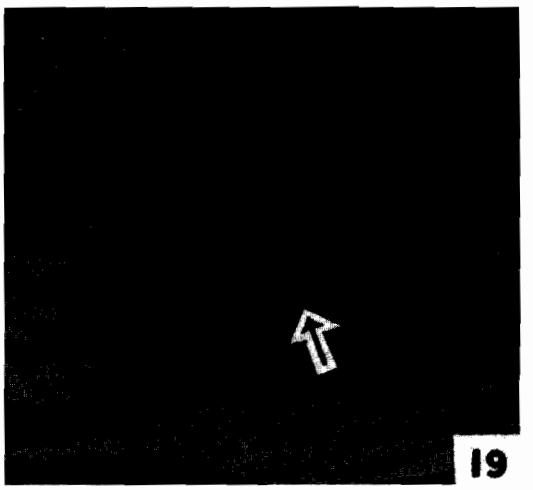

Fig 19–72 hours after challenge in immunized mice, showing disappearance of AKP in the cuticle (arrow) of dead larva in granuloma located in abdominal muscle x 200 Gomori's calcium cobalt method.

Table 1
Histochemical alteration of challenged *Ancylostoma caninum* infected third-stage larvae (L_3) in mice immunized with L_3.

Histochemical substances of L_3 (content or enzyme activity)	Time after challenge (hours)	Reduction of histochemical substances of challenged L_3 (%)					
		Immunized mice			Non-Immunized mice		
		L_3SE	N (%)	R (%)	Dis (%)	L_3SE	N (%)
Glycogen	6	167	74 (44.3)	70 (41.9)	23 (13.8)	21	21 (100)
	24	199	73 (36.7)	76 (38.2)	50 (25.1)	21	21 (100)
	72	126	12 (9.5)	33 (26.2)	81 (64.3)	42	42 (100)
RNA	6	135	120 (88.9)	15 (11.1)	0 (0)	26	26 (100)
	24	164	144 (87.8)	20 (12.2)	0 (0)	33	33 (100)
	72	148	92 (62.2)	52 (35.1)	4 (2.7)	5	5 (100)
DNA	6	130	66 (50.8)	60 (46.2)	4 (3.0)	47	47 (100)
	24	118	76 (64.4)	34 (28.8)	8 (6.8)	8	8 (100)
	72	140	60 (42.9)	60 (42.9)	20 (14.2)	8	8 (100)
Alkaline protein	6	196	126 (64.3)	70 (35.9)	0 (0)	26	26 (100)
	24	155	110 (71.0)	40 (25.8)	5 (3.2)	16	16 (100)
	72	126	54 (42.9)	48 (38.1)	24 (19.0)	4	4 (100)
Acid aucopoly-	6	140	51 (36.4)	84 (60.0)	5 (3.6)	28	28 (100)
sacharride	24	196	71 (36.2)	105 (53.6)	20 (10.2)	17	17 (100)
	72	136	0 (0)	96 (70.6)	40 (29.4)	6	6 (100)
Collagen	6	120	60 (50.0)	60 (50.0)	0 (0)	36	36 (100)
	24	135	55 (40.7)	65 (48.1)	15 (11.1)	6	6 (100)
	72	106	40 (37.7)	48 (45.3)	18 (17.0)	6	6 (100)
Reticulin	6	140	85 (60.7)	40 (28.6)	15 (10.7)	18	18 (100)
	24	135	45 (33.3)	60 (44.4)	30 (22.2)	11	11 (100)
	72	120	24 (20.0)	60 (50.0)	36 (30.0)	1	1
Alkaline	6	168	56 (33.3)	56 (33.3)	56 (33.3)	44	44 (100)
phosphatase	24	150	30 (20.0)	60 (40.0)	60 (40.0)	14	14 (100)
	72	160	16 (10.0)	60 (37.5)	84 (52.2)	4	4 (100)
Adenosine	6	138	30 (21.7)	84 (60.9)	24 (17.4)	25	25 (100)
triphosphatase	24	148	18 (12.2)	72 (48.6)	58 (39.2)	34	34 (100)
	72	132	16 (12.1)	32 (24.2)	84 (63.6)	6	6 (100)

L_3SE : L_3 sections examined; N: normal; R: reduction; Dis: disapearance.

18,19). The percentage of normal appearing L_3 decreased over the interval between 6-72 hours post-challenge. In contrast, there was no loss in enzyme activity found in L_3 contained in sections from non-immunized mice. The quantitative differences are summarized in Table 1. At 7-14 days the challenge L_3 which survive the immune response appear to have normal enzyme staining.

DISCUSSION

We have shown previously that vaccination of mice with L_3 of *A.caninum* results in both humoral and cell-mediated immune responses (Xiao *et al*, 1998a, b; 1999; Xue *et al*, 1999; Yang *et al* 1998; Yang *et al*, submitted; Ghosh *et al*, 1996; Ghosh and Hotez, submitted) directed against invading larvae administered in a challenge dose. Anti-L_3 humoral responses are comprised of high levels of total IgE as well as elevations in parasite-specific IgM (Ghosh and Hotez, submitted). Anti-L_3 cellular inflammatory responses are comprised of several different cell types including eosinophils and mast cells (Yang *et al*, submitted), which in some cases form granulomata surrounding the invading parasite. Both humoral and cellular inflammatory responses may operate by an antibody dependent cell mediated cytotoxicity mechanism (Xiao *et al*, 1998b). Our studies here were very similar to the histochemical

appearance of other nematodes seen in tissue sections (Anga, 1966; Yang *et al*, 1988; 1989a, b; 1996), but go on to show that immune-mediated destruction of challenge L_3 is always associated with histological degeneration and histochemical alteration of parasite structural components. There is also biochemical evidence for loss in parasite enzyme activity.

L_3-vaccination results in a high level of protection against larval challenge when measured in terms of reduction in worm burden. However, L_3-vaccination does not result in sterilizing immunity (Xiao *et al*, 1998a) and therefore some larvae survive host immune and inflammatory responses have a normal histological appearance.

There is another possible interpretation of our findings. The reduction in histochemical content of L_3 from tissue section of vaccinated mice could reflect altered metabolic function of the parasite. This could also account for the changes noted in parasite AKP and ATPase activities, as well as increased glycogen consumption. This interpretation is consistent with the old concept of "ant-enzyme antibodies" first espoused by Chandler over 60 years ago (Chandler, 1932). Similarly, the normal appearance of L_3 which survive challenge could be associated metabolic recovery. It may be possible to test this hypothesis *in vitro* by administering mouse ant-L_3 antibodies to *A.caninum* L_3 both in the presence and absence of immunocompetent cells. Hookworm L_3 treated in this manner could then be evaluated histochemically.

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