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The Role of L3T4$^+$ T Lymphocytes in the Development of Protective Immunity to *Hymenolepis nana* in Mice

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Abstract

The role of L3T4$^+$ T cells in protective immunity to *Hymenolepis nana* was studied in BALB/c mice that were injected with anti-L3T4 monoclonal antibody. All mice injected twice at the 1-week interval with antibody (0.5 mg/injection) showed marked reduction of protective immunity; all were positive for cysticercoids from challenge infection. Anti-L3T4 monoclonal antibody treatment completely eliminated the L3T4$^+$ T cells, and inhibited the increase in numbers of mesenteric lymph node cells (MLNC) that is characteristically induced by *H. nana* infection. Anti-L3T4 monoclonal antibody also completely blocked the proliferative response of MLNC to *H. nana* egg antigen.

Key words: *Hymenolepis nana,* BALB/c mouse, protective immunity, L3T4$^+$ T lymphocytes, depletion of T-cell subsets *in vivo*

Introduction

A wide variety of experimental evidence has demonstrated that protective immunity to *Hymenolepis nana* in the mouse is a strongly thymus-dependent phenomenon (Okamoto, 1968; Okamoto, 1970; Reed *et al.*, 1977; Asano *et al.*, 1982). Using cell-depletion and adoptive transfer techniques, we have demonstrated that Lyt-1$^+$ T cells play a major role in protective immunity against cysticercoids (Asano *et al.*, 1986). However, it is reported that Lyt-1 antigen is expressed by not only all T cells (Ledbetter *et al.*, 1981) but also some B cells (Lanier *et al.*, 1981; Manohar *et al.*, 1982). Different from Lyt-1 antigen, L3T4 antigen is expressed only by T cells. L3T4 and Lyt-2 antigens are expressed by mutually exclusive T-cell subsets so that all T cells express either L3T4 antigen or Lyt-2 antigen (Dialynas *et al.*, 1983). Judging from these reports, it is conceivable that L3T4 antigen is more relevant than Lyt-1 antigen for T-cell subsets. In the *H. nana*-mouse system, relations between L3T4$^+$ T cells and protective immunity remains to be established. Recent studies indicate that *in vivo* administration of monoclonal antibody to L3T4 antigen selectively eliminates T cells bearing this surface antigen (Cobbold *et al.*, 1984). In this paper, we evaluated the role of L3T4$^+$ T cells in protective immunity against *H. nana* in mice by using *in vivo* administration of anti-L3T4 monoclonal antibody.

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Materials and Methods

Mice

Male BALB/c mice used in this study were bred and maintained in our laboratory from a breeding colony originally obtained from Charles River Japan, Inc. They were maintained under conventional laboratory conditions.

Parasite

The parasite utilized was *H. nana* maintained routinely in our laboratory. The methods used for preparation of egg suspension and for oral inoculation with eggs were described previously (Okamoto, 1968).

Soluble egg antigen

The method for preparation of soluble egg antigen of *H. nana* has been described previously (Asano *et al.*, 1990). Briefly, *H. nana* eggs suspended in 0.05 M phosphate-buffered saline were homogenized with a sonic dismembrator (60 Watt; Artek Systems, Corp., N.Y., USA) for 1 hr at 4°C. The homogenate was centrifuged at 16,000 *g* for 1 hr at 4°C. The supernatant fluid was assayed for protein concentration using the method of Bradford (1976) and finally adjusted to 200 μg/ml.

Thymectomy

Mice were thymectomized at 4 weeks of age according to the technique of Miller (1960) to remove a source of T lymphocytes. Before the operation they were injected intraperitoneally with 0.2 ml of 0.5 mg/ml pentobarbital sodium (Nembutal; Abott Lab., IL., USA) and were subsequently anesthetized by ether. After surgery they were revived under a warm lamp.

Monoclonal antibody

The monoclonal antibodies used in this study were mouse Ig G2b antibody against the Thy-1.2 antigen, mouse Ig M antibody against the Lyt-2.2 antigen and rat Ig G2b antibody against the mouse L3T4 antigen. These antibodies were purchased from Cedarlane Laboratories, Ltd., Canada. The minimum amount of antibody to cause maximum killing of lymph node cells was 0.2 μg/5 $\times$ 10^6 cells for anti-Thy-1.2, 0.1 μg/1 $\times$ 10^7 cells for anti-Lyt-2.2, and 2.0 μg/l $\times$ 10^7 cells for anti-L3T4.

In vivo treatment with antibody

After 3 weeks of thymectomy the mice were injected twice at the 1-week interval with either 0.5 mg (0.25 ml) of anti-L3T4 monoclonal antibody or 0.25 ml of saline (Cobbold *et al.*, 1984).

Immunization with H. nana and estimation of protective immunity

Two weeks after the final antibody injection, antibody-treated and control mice were immunized by an oral inoculation with 1,000 eggs. To estimate protective immunity, they were challenged with 1,000 eggs 5 days after immunization and were killed 4 days after the challenge. The small intestine was removed and examined for adult worms, and for cysticercoids according to the methods described previously (Okamoto, 1968).

Cell suspension and analysis of cell number

Suspension of mesenteric lymph node cells (MLNC) was prepared from individual mice according to the method described by Asano *et al.*, (1986), with the exception that all procedures were carried out in RPMI-1640 (HAZLETON, PA., USA) supplemented with 10% foetal calf serum (FCS: HAZLETON), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 $\times$ 10^{-5}M 2-ME and 10 mM HEPES (RPMI-FCS). Red blood cells were lysed by suspending 2 $\times$ 10^7 cells in 10 ml of 0.75% NH_4Cl-0.15 M Tris for 5 min. The residual cells were suspended in a known volume of RPMI-FCS and a total cell number was determined with a haemocytometer in the presence of 0.2% trypan blue. The number of lymphoid cells expressing Thy-1.2, L3T4 or Lyt-2 markers was assessed by the antibody-dependent cytotoxicity assay described by Gorer and O'Gorman (1956). Briefly, 5 $\times$ 10^6 cells in 1 ml RPMI-FCS were incubated at 37°C for 30 min with 0.1 mg of the appropriate antibody. The cell suspensions were then centrifuged at 200 *g* for 10 min at room temperature. The cell pellet was resuspended in 1 ml of 1:10 diluted Low-Tox Rabbit Complement (Cedarlane Lab., Ltd., Canada). After incubation at 37°C for a further 45 min, the cells were washed twice with RPMI-FCS and were resuspended in 1 ml fresh medium. The percentage of killed cells was determined by trypan blue staining and then the net percentage of killed cells was calculated according to the following formula: Net % of killed cells = {(number of cells killed by antibody + complement – number of cells killed by complement alone)/100 – number of cells killed by complement alone} $\times$ 100. The absolute number of either Thy-1.2$^+$, L3T4$^+$, or Lyt-2$^+$ cells was calculated by multiplying their net percentage

value by total yield of cells.

Proliferative response of MLNC

The method used for examination of proliferative response was as described previously (Asano *et al.*, 1990). The essential features are as follows: MLNC from five different mice were pooled and were resuspended at a concentration of 5×10^6 cells/ml. Aliquots containing 5×10^5 cells were introduced into each well of a 96-well flat-bottomed microculture plate (NUNC, Denmark) that contained 0.1 ml (200 μg/ml) of soluble egg antigen. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 hr of culture, 1.0 μCi ^{3}H-thymidine (^{3}H-TdR; Spe. Act. 20.0 Ci/m mol: NEN Corp., MA., USA) was added to each well and cultured for a further 24 hr. The cells were then harvested onto glass fiber filter discs and ^{3}H-TdR incorporation was measured in a Packard Tricarb Liquid Scintillation Spectrometer. The results were expressed as the mean counts per minute (cpm) for triplicate cultures plus or minus SD.

Results

Effect of anti-L3T4 monoclonal antibody treatment on protective immunity

Dependence of development of protective immunity on the presence of L3T4$^+$ T cells was evaluated. Antibody-treated mice, together with untreated and saline-treated controls, were orally inoculated with *H. nana* eggs and were challenged on the 5th day after the immunizing infection. Mice were sacrificed 4 days after the challenge and the intestinal villi examined for cysticercoids. Control mice had strong capacity for rejecting cysticercoids; all were negative for cysticercoids derived from the challenge infection. In contrast, mice treated with anti-L3T4 monoclonal antibody failed to reject cysticercoids. All mice gave evidence of reinfection and harbourd intestinal cysticercoids (Table 1).

Effects of anti-L3T4 monoclonal antibody treatment on total cell numbers and numbers of T-cell subsets in the mesenteric lymph nodes of control and H. nana-infected mice.

To investigate the effects of anti-L3T4 monoclonal antibody treatment on alteration of total cell numbers and numbers of T-cell subsets in mesenteric lymph nodes, the numbers of lymphocytes expressing the Thy-1.2, L3T4 or Lyt-2 surface antigen were determined. MLNC were prepared from uninfected mice and from mice that were orally inoculated with eggs 5 days before. Anti-L3T4 monoclonal antibody treatment caused a marked decrease in the total numbers of MLNC detected in uninfected mice, and inhibited the increase in number of MLNC that is characteristically induced by *H. nana* in-

Table 1 Effect of *in vivo* treatment with anti-L3T4 monoclonal antibody on the protective immunity to *Hymenolepis nana* in BALB/c mice

Treatment	No. reinfected / No. challenged	No. of cysticercoids in individual mice
None	0/3	0 0 0
Saline	0/3	0 0 0
Anti-L3T4 monoclonal antibody	6/6	133 134 175 180 218 290

BALB/c mice were thymectomized at 4 weeks and randomly selected 3 weeks later for antibody administration, given 1 week apart as two intravenous injection of 0.5 mg (0.25 ml) antibody. Two weeks later, mice were orally immunized with 1,000 eggs, and then challenged 5 days after the immunization. Mice were sacrificed 4 days after the challenge and the intestinal villi were examined for cysticercoids.

Table 2 Cellular changes in the mesenteric lymph nodes of BALB/c mice pretreated with anti-L3T4 monoclonal antibody.

H. nana Infection	Anti-L3T4 Injection	No. of cells ($\times 10^6$)			
		Total	Thy-1.2$^+$	L3T4$^+$	Lyt-2$^+$
−	−	15.0 ± 1.3	8.5 ± 1.3	6.8 ± 0.6	1.8 ± 0.4
−	+	3.0 ± 1.1	2.1 ± 0.2	0	2.1 ± 0.3
+	−	40.2 ± 3.3	26.7 ± 2.6	14.3 ± 7.5	5.3 ± 0.8
+	+	7.0 ± 0.9	2.7 ± 0.4	0	2.1 ± 0.4

BALB/c mice (5 mice/group) were thymectomized at 4 weeks and randomly selected 3 weeks later for anti-L3T4 monoclonal antibody (Anti-L3T4) administration, given 1 week apart as two intravenous injections of 0.5 mg (0.25 ml) antibody. Two weeks later, mice were orally immunized with 1,000 *H. nana* eggs, and killed 5 days after the immunization.

fection (Table 2). The effect of anti-L3T4 monoclonal antibody treatment on T-cell subsets of MLNC was studied by determining the absolute numbers of cells bearing surface phenotypes of Thy-1.2, L3T4, or Lyt-2. The data in Table 2 also demonstrated that injection of mice with anti-L3T4 monoclonal antibody completely eliminated the L3T4$^+$ MLNC. However, this treatment of mice did not cause a decrease in the number of Lyt-2$^+$ MLNC.

Effect of anti-L3T4 monoclonal antibody treatment on in vitro proliferative response of MLNC.

To examine the effect of anti-L3T4 monoclonal antibody treatment on the proliferative response of MLNC to egg antigen, MLNC were cultured *in vitro* in the presence or absence of egg antigen. The responsiveness of MLNC was examined by means of a ^{3}H-TdR incorporation system. *In vivo* treatment with anti-L3T4 monoclonal antibody completely blocked the proliferative response of MLNC to egg antigen. The incorporation of ^{3}H-TdR by MLNC showing cell proliferation, which averaged 7,647 cpm in uninfected mice, increased to 128,318 cpm in infected mice but failed to increase in infected mice treated with anti-L3T4 monoclonal antibody (Table 3).

Discussion

A number of experimental approaches have

Table 3 Effect of anti-L3T4 monoclonal antibody treatment on the proliferative response of mesenteric lymph node cells to soluble egg antigen of *Hymenolepis nana*.

H. nana Infection	Anti-L3T4 Injection	Culture medium containing	
		None	Egg Antigen
−	−	7,211 ± 961	7,647 ± 568
−	+	5,121 ± 342	5,242 ± 602
+	−	15,920 ± 176	128,318 ± 168
+	+	6,667 ± 51	12,847 ± 79

BALB/c mice (5 mice/group) were thymectomized at 4 weeks and randomly selected 3 weeks later for anti-L3T4 monoclonal antibody (Anti-L3T4) administration, given 1 week apart as two intravenous injections of 0.5 mg (0.25 ml) antibody. Two weeks later, mice were orally immunized with 1,000 *H. nana* eggs, and killed 5 days after the immunization. Mesenteric lymph node cells were cultured for 48 hr at a concentration of 5×10^5 cells in the presence of soluble egg antigen. Values expressed here as the mean counts per minute for triplicate cultures ± SD.

demonstrated the importance of T cells for the establishment of protective immunity to *H. nana* (Uchida *et al.*, 1982; Asano *et al.*, 1986). It is also established that T cells are not a homogeneous population, but consist rather of distinct subsets, which can be defined in terms of unique cell surface markers, e.g. the Lyt antigens and the L3T4 antigen (Cantor and Boyse, 1976; Dialynas *et al.*, 1983). Although a previous report from our laboratory showed that T cells that initiate protective immunity to *H. nana* express the Lyt-1 antigen (Asano *et al.*, 1986), it is not determined whether the $L3T4^+$ T cells are required for development of protective immunity.

The present results clearly demonstrated that $L3T4^+$ T cells of MLNC are strongly implicated in initiating protective immunity, and most cells that respond to *H. nana* antigen express L3T4 antigen on their cell surface. These conclusions are supported by the following observations: First, in contrast to the results obtained from control mice, mice treated with anti-L3T4 monoclonal antibody were all positive for cysticercoids derived from challenge infection. Second, administration of anti-L3T4 monoclonal antibody was resulted in a dramatic decrease in the number of $L3T4^+$ T cells, but not in that of $Lyt\text{-}2^+$ T cells. Third, administration of anti-L3T4 monoclonal antibody completely blocked the incorporation of ^{3}H-TdR by MLNC that showed a proliferative response of cells to egg antigen.

Although the precise mechanisms by which $L3T4^+$ T cells mediate the process of worm rejection is not fully understood, there is some circumstantial evidences that acute inflammatory responses mediated by $L3T4^+$ T cells (Mosmann and Coffman, 1989) cause expulsion of worms from the small intestine (Urquhart *et al.*, 1965; Larsh and Race, 1975; Wakelin and Wilson, 1979). Miyazato *et al.* (1979) reported that, in the *H. nana*-mouse system, an acute inflammatory response was initiated around the oncospheres 4 hours after the challenge and reached a zenith at about 24 hours. From the present results, it is reasonable to propose that sensitized $L3T4^+$ T cells in the mesenteric lymph nodes proliferate in response to *H. nana* antigen, and release a variety of lymphokines that may be involved in amplification and differentiation of inflammatory responses. Indeed, our previous report (Asano *et al.*, 1990), and unpublished data have shown that sensitized MLNC proliferating in response to soluble egg antigen release lymphokines such as interleukin (IL)-1, IL-2, IL-3 and γ-interferon. The accumulation of eosinophils in the intestinal wall (Miyazato *et al.*, 1979; Friedberg *et al.*, 1979) and in peripheral blood (Shimoda *et al.*, 1982) may reflect lymphokine release by specifically activated MLNC. Furthermore, granuloma formation around the oncospheres (Miyazato *et al.*, 1979) may also reflect lymphokine production, because IL-1 is important in the initiation and maintenance of granuloma formation (Kasahara *et al.*, 1988). Further investigation in this direction will provide valuable information concerning the cellular mechanisms involved in worm expulsion from the small intestine.

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