[Jpn. J. Parasitol., Vol. 39, No. 1, 28–35, February, 1990]

Protective Activity of Secondary Effector Lymphocytes Generated *in vitro* to *Hymenolepis nana* Infection *in vivo* and An Assay for *in vitro* Lymphocyte Proliferation

KAZUHITO ASANO, KUNIHIKO MURAMATSU AND KEN-ICHI OKAMOTO

(Accepted for publication; October 31, 1989)

Abstract

Mesenteric lymph node cells (MLNC) from *Hymenolepis nana*-infected mice were cultured *in vitro* with *H. nana*-soluble egg antigen. MLNC profusely proliferated when cultured in the presence of egg antigen, as assessed by measuring ^{3}H-thymidine incorporation. The transfer of MLNC, prepared from donor mice infected 10 days before cell collection and cultured with egg antigen, conferred strong protective immunity on syngeneic recipient nude mice as judged by complete rejection of cysticercoids. The transfer of MLNC cultured without egg antigen had no demonstrable effect on cysticercoid rejection. The culture supernatant contained detectable levels of interleukin 2 (IL-2) and macrophage activating factor, but no macrophage migration inhibition factor. MLNC responded well and proliferated in the presence of an exogenous IL-2. These results strongly suggest that *in-vitro* culture of MLNC with soluble egg antigen produces the expansion of cells that are responsible for protective immunity, and intravenous administration of cultured cells brings about complete rejection of cysticercoids in recipient nude mice.

Key words: *Hymenolepis nana,* mouse, mesenteric lymph node cells, soluble egg antigen, interleukin 2, *in vitro*

Introduction

It is well accepted that primary infection of mice with *Hymenolepis nana* eggs evokes the development of strong cell-mediated immunity that brings about the complete rejection of cysticercoids derived from the challenge infection (Heynemann, 1963, Okamoto, 1970). Previous reports from our laboratory showed: 1) This state of immunity was adoptively transferable to athymic nude mice with short-term sensitized mesenteric lymph node cells (MLNC), but not with long-term sensitized MLNC (Asano *et al.*, 1986). 2) There is a temporal correlation between the high blast cell activity in the mesenteric lymph nodes and capacity of these blastoid cells to adoptively transfer immunity (Asano *et al.*, 1988). 3) Mesenteric lymph nodes from mice infected with *H. nana* contain an enlarged population of rapidly dividing T cells (Asano *et al.*, 1988). Such studies lead to the hypothesis that T cells in the mesenteric lymph nodes become activated and transform rapidly into blast cells when encountered by antigens, and these blastoid cells result in complete rejection of cysticercoids in the intestinal villi of infected mice. To additionally clarify these two hypotheses, we investigated the reactivity of MLNC to *H. nana* egg antigen.

Department of Medical Biology, School of Medicine, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142, Japan
浅野和仁　村松邦彦　岡本謙一（昭和大学医学部医動物学教室）

Materials and Methods

Animals

Congenitally athymic nude mice, progeny of the BALB/c strain, and normal BALB/c mice were purchased from CLEA, Japan Inc., Tokyo. All mice were male and 5 weeks of age at the beginning of the experiments. Experimental and control groups each consisted of 6 mice, unless stated otherwise.

The parasite used in this study was *H. nana*. The methods used for preparation of egg suspension and for oral inoculation with eggs have been

described previously (Okamoto, 1968).

Soluble egg antigen

To obtain soluble egg antigen, *H. nana* eggs suspended in 0.05 M PBS (pH 7.0) were homogenized with a 60 W sonic dismembrator (Artek Systems Co., Farmingdale, N.Y., USA) for 60 min in an ice bath. The homogenate was clarified by centrifugation at 16,000 *g* for 60 min at 4°C. The protein concentration of the supernatant was determined by measuring the optical density at 280 nm and adjusted to 20 mg/ml. The solution was filtered through a Millipore filter (0.22 μm; Nihon Millipore Kogyo K. K., Yonezawa, Japan) and stored at −40°C until used.

Cell suspension

Suspensions of MLNC in RPMI-1640 (Flow Lab., North Ryde, Australia) supplemented with 10 mM HEPES, 1.0 mM sodium pyruvate, 5×10^{-5}M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum (Flow Lab.) were prepared by the method described previously (Asano *et al.*, 1986).

Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity of non-infected mice with the medium on day 3 after intraperitoneal injection with 1.0 ml of 10% proteose peptone (Difco Lab., Detroit, Mich., USA).

Cell culture

To examine the *in-vitro* lymphocyte transformation, MLNC were cultured with or without egg antigen. Aliquots of 5×10^5 MLNC (0.1 ml) were placed in each well of a 96-well flat-bottomed microculture plate (Nunc, Denmark) containing 0.1 ml of egg antigen or the medium. The plate was then maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. After 24 hr the cells were pulsed for another 24 hr with 1.0 μCi of ^{3}H-thymidine (^{3}H-TdR; Spe. act. 20 Ci/mM; New England Nuclear, Boston, Mass., USA). The uptake of ^{3}H-TdR by MLNC was measured in a Packard Tricarb liquid scintillation spectrometer. The results were expressed as mean counts per minute (cpm) plus or minus standard deviation of triplicate cultures of individual experiments.

To prepare the cells used for transfer, MLNC were cultured by a method similar to that described above. These cells were harvested on day 4 of the culture and washed 5 times before transfer to recipients.

Response to exogenous interleukin 2

Response to interleukin 2 (IL-2) was examined by proliferation assay of MLNC in the presence of exogenous IL-2, according to the method described by Wofsy *et al.* (1981). Briefly, 5×10^5 MLNC (0.1 ml) was introduced into each well of a 96-well flat-bottomed microculture plate (Nunc) containing 0.1 ml of Con A-free IL-2 (Immuno. Biol. Lab., Fujioka, Japan; Lot No. Rt-5). The plate was maintained for 48 hr at 37°C and 5% CO_2, ^{3}H-TdR (0.5 μCi/well) was added during the last 24 hr of the 48-hr culture. They were then harvested and the incorporation of ^{3}H-TdR was determined.

Culture Supernatant

MLNC were cultured in a 24-well flat-bottomed microculture plate (Becton Dickinson Co., Sunnyvale, Calif., USA) at a concentration of 5×10^6 cells/well, with or without egg antigen, for 24 hr. The culture supernatants were collected by centrifugation at 1,600 *g* for 10 min at room temperature and stored at −40°C until used.

Assay for Lymphokine

The lymphokines examined in this study were IL-2, macrophage activating factor (MAF) and macrophage migration inhibition factor (MIF).

IL-2 activity in the supernatants was assayed by the ability to support the proliferation of the IL-2 dependent cell line, CTLL-2 (Gillis *et al.*, 1978). The uptake of ^{3}H-TdR by CTLL-2 in response to serial two-fold dilution of the test samples was compared to the response of a standard rat IL-2 preparation (Immuno. Biol. Lab., Lot No. Rt-5) by probit analysis (Gillis *et al.*, 1978). The activity of IL-2 in test samples was expressed as U/ml, as given by the following formula: U/ml = 100 × (reciprocal titer of test sample at 50% of maximal cpm of standard/

reciprocal titer at 50% of maximal cpm of standard).

MAF activity in the supernatants was assayed by the capacity to activate PEC to inhibit the growth of EL-4 tumor cells, using the method based on those described by Kumazawa *et al.* (1984). The results were expressed as percent inhibition of EL-4 growth, as given by the following formula: % inhibition = 100 − {(cpm test group − cpm PEC only)/cpm EL-4 only} × 100.

MIF activity in the culture supernatants was examined by the capillary tube method using PEC as indicator cells. The results were expressed as migration index (MI), as given by the following formula: MI(%) = {(average migration of cells in the supernatant/average migration of cells in RPMI-1640 medium)} × 100.

Cell transfer and assay for protection

Cultured cells were injected intravenously and adoptive immunity estimated as described previously (Asano *et al.*, 1986).

Results

Proliferative response of MLNC to H. nana egg antigen

MLNC were prepared from 6 individual BALB/c mice infected 4 days previously with 1,000 *H. nana* eggs (4 Day MLNC), and cultured *in vitro* with egg antigen. MLNC from 4 individual non-infected mice were similarly cultured. To examine the optimal dose of egg antigen for stimulation of MLNC, 4 Day MLNC were cultured with various concentration of egg antigen. The results of the experiments are illustrated in Fig. 1. MLNC from infected mice responded to egg antigen at concentrations of 50 to 1,300 μg/ml. Maximal proliferation of MLNC was observed at a concentration of 200 μg/ml. The incorporation of ^{3}H-TdR showed a suppression or a plateau when MLNC were cultured with egg antigen at concentrations of more than 300 μg/ml. MLNC from non-infected mice responded only slightly to egg antigen (Fig. 1).

To examine the specificity of the proliferative response, 4 Day MLNC were cultured with either egg antigen, human-γ-globulin (HGG; Miles Lab., Inc., Naperville, IL., USA) or ovalbumin (OVA; Boehlinger Mannheim GmbH, W. Germany), at concentrations of 200 μg/ml. As shown in Fig. 2, 4 Day MLNC did not proliferate in response to HGG or OVA.

The kinetics of the MLNC proliferative response to egg antigen was examined by using cells prepared on various days post-infection. MLNC were prepared from BALB/c mice infected with 1,000 *H. nana* eggs on days either 4, 10, 21 or 30 post-infection and the cells were pooled for each group. The pooled cells were cultured with 200 μg/ml of egg antigen. Control cells were prepared from non-infected mice and cultured in the same manner. The results are shown in Table 1. The strong proliferative responses of MLNC were observed on all days post-infection examined and % increase in cpm similar on all days. However, MLNC prepared from non-infected mice did not exhibit the proliferative response to egg antigen. This experiment was performed 3 times with nearly identical results.

Transfer of protective immunity to nude mice

The ability of MLNC, cultured with egg antigen, to transfer immunity into nude mice was tested. MLNC were prepared from donor mice infected with 1,000 *H. nana* eggs 10 days before and cultured for 4 days. Two groups of mice were given 5×10^7 cultured MLNC on days 6, 4 and 2 prior to the challenge. Together with controls (4 mice), the recipients were challenged with 1,000 *H. nana* eggs on day 0 and killed 4 days later. As shown in Table 2, all the recipients of group 1 (uninjected control) gave evidence of infection and harbored intestinal cysticercoids ranging from 141 to 190 per mouse. A similar result was obtained in group 2, in which mice received MLNC cultured without egg antigen and were all positive for cysticercoids. Adoptive immunity was successful in 5 out of 6 mice of group 3, in which mice received MLNC cultured with egg antigen. The remaining one mouse of this group harbored only two cysticercoids.

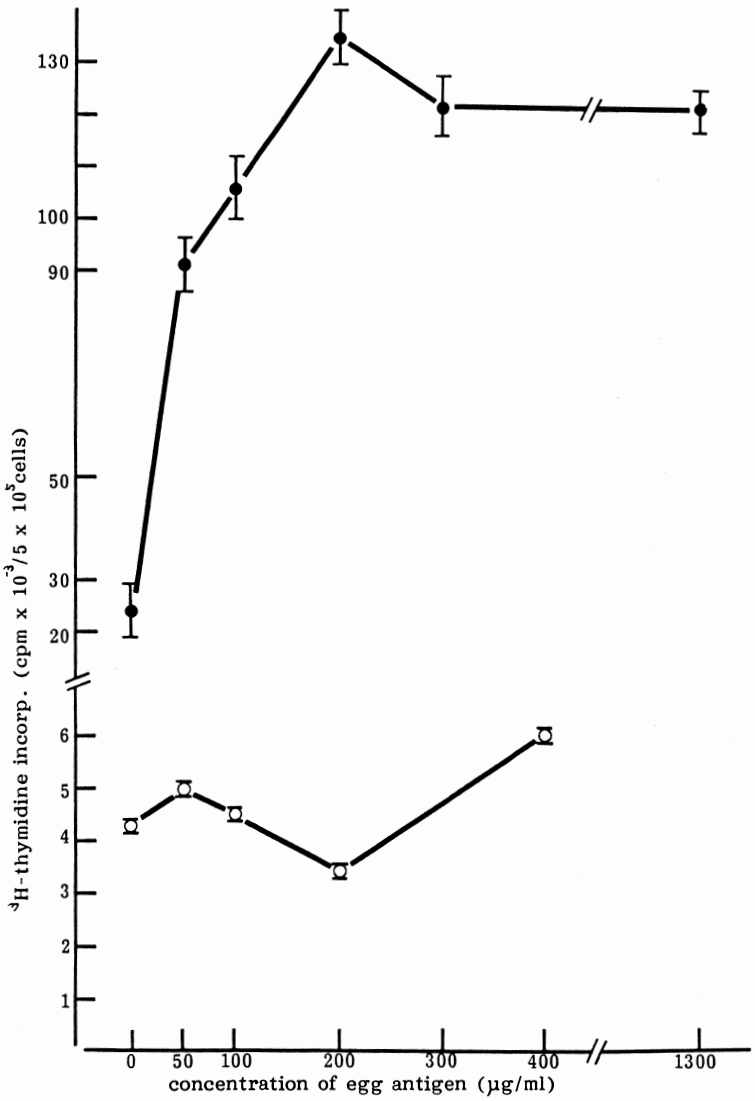
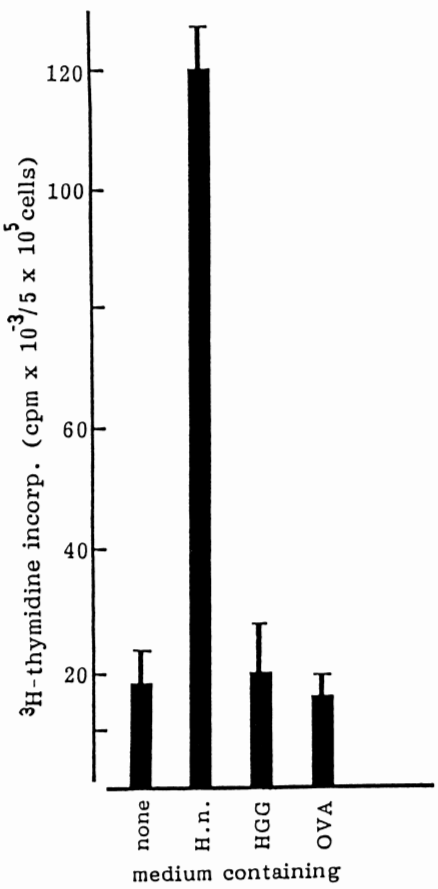


Fig. 1. Dose response profile of the proliferation of mesenteric lymph node cells (MLNC). MLNC from BALB/c mice infected with *H. nana* 4 days previously (●) and non-infected mice (○) were cultured *in vitro* with various concentration of soluble egg antigen of *H. nana*.

In vitro lymphokine production

The ability of MLNC to release lymphokines into the surrounding culture medium in *in vitro* culture with 200 μg/ml egg antigen was examined. To accomplish this, 4 groups of BALB/c mice were infected with 1,000 *H. nana* eggs on day 0. MLNC were prepared on days 4, 10, 21 and 30 post-infection and pooled for each group. Control cells were also prepared from non-infected mice. The results are shown in Table 3. No detectable levels of IL-2 were present in the supernatant of MLNC from non-infected mice cultured with or without egg antigen. However, when MLNC from infected mice were cultured with egg antigen, the culture supernatant did contain a detectable level of IL-2.



Similar levels of MAF were detected in the supernatants of MLNC from non-infected mice cultured with or without egg antigen. In contrast, high levels of MAF were detected in the supernatants of MLNC from infected mice when cells were cultured with egg antigen. Inhibition of macrophage migration, showing the presence of MIF, was not observed when PEC were cultured in the supernatant. This experiment was performed twice with similar results.

Response to exogenous IL-2

The IL-2 response of MLNC prepared from *H. nana*-infected mice was examined. To determine the IL-2 dose used in this study, MLNC from non-infected mice were cultured with various concentration of Con A free IL-2 and ^{3}H-TdR incorporation was examined. Con A free IL-2 was used in a final concentration of 1:2 while examining the proliferative response to IL-2 in infected mice, since this dose produced a

Fig. 2. Specificity of the proliferative response of MLNC. Mesenteric lymph node cells (MLNC) were prepared from BALB/c mice infected with *H. nana* 4 days previously. MLNC were cultured *in vitro* with either *H. nana* egg antigen (H. n.), Human-γ-globulin (HGG) or Ovalbumine (OVA).

Table 1 Antigen-induced proliferation of mesenteric lymph node cells prepared from BALB/c mice infected with *Hymenolepis nana*

Days post infection	Egg antigen	^{3}H-thymidine incorporation	
		cpm ± S.D.	% cpm ± S.D.
–	–	4690 ± 1269	100
(non-inf.)	+	3510 ± 459	79 ± 27
4	–	29200 ± 2621	100
	+	120562 ± 6972	493 ± 35
10	–	15729 ± 1605	100
	+	76760 ± 2703	495 ± 48
21	–	11579 ± 634	100
	+	72129 ± 3233	605 ± 27
30	–	13238 ± 495	100
	+	70431 ± 3003	560 ± 36

5×10^5 MLNC were exposed *in vitro* to 200 μg of egg antigen per ml for 48 hr. Stimulation was measured by the degree of incorporation of ^{3}H-thymidine.

Table 2 Degree of immunity of mesenteric lymph node cells cultured with or without *Hymenolepis nana* egg antigen in nude mouse recipients

Group No.	Type of donor cells	No. of donor cells	No. of recipients examined	No. of cysticercoids in in individual recipients
1	–(Uninjected control)	–	4	141 170 176 190
2	immune MLNC cultured without egg antigen	1.5×10^8	6	71 115 134 149 260 261
3	immune MLNC cultured with egg antigen	1.5×10^8	6	0 0 0 0 0 2

Mesenteric lymph node cells (MLNC) were collected from BALB/c mice immunized by oral administration with 1,000 *H. nana* eggs 10 days before cell collection.

Table 3 Lymphokine activity in supernatants prepared from *in vitro* cultures using mesenteric lymph node cells from BALB/c mice infected with *Hymenolepis nana*

Days post infection	Egg antigen	Lymphokine*		
		IL-2 (U/ml)	MAF (% inhibition of EL-4 growth)	MIF (Migration index)
–	–	N.D.	35.3	98.8
(non-inf.)	+	N.D.	36.1	109.0
4	–	N.D.	38.5	96.9
	+	8.0	94.5	105.5
10	–	N.D.	N.T.	N.T.
	+	4.6	94.6	N.T.
21	–	N.D.	38.4	106.9
	+	5.2	78.4	109.4
30	–	N.D.	35.1	N.T.
	+	6.0	79.6	N.T.

* : The activities are the mean of duplicate determinations.
IL-2 : interleukin 2, MAF: macrophage activating factor
MIF : macrophage migration inhibition factor
N.D. : Not Detected, N.T.: Not Tested

significant proliferative response of MLNC (data not shown). MLNC were prepared from 3 individual non-infected mice, and 5 individual mice infected with 1,000 *H. nana* eggs on days 4, 10 and 21 post-infection. As shown in Table 4, MLNC from non-infected mice respond poorly to added IL-2, whereas MLNC from infected mice respond vigorously. This experiment was performed twice with similar results.

Table 4 Response to exogenous interleukin 2 of mesenteric lymph node cells prepared from BALB/c mice infected with *Hymenolepis nana*

Days post infection	Culture medium containing IL-2	^{3}H-thymidine uptake (cpm ± S.D.)	% increase
–	–	4561 ± 163	100
(non-inf.)	+	16181 ± 1347	354.8 ± 29.5
4	–	18165 ± 1297	100
	+	112404 ± 4046	592.7 ± 19.0
10	–	13630 ± 6186	100
	+	133987 ± 1574	983.0 ± 24.2
21	–	13811 ± 1469	100
	+	116810 ± 2335	845.7 ± 15.1

5×10^5 mesenteric lymph node cells were cultured *in vitro* with or without interleukin 2 (IL-2) for 48 hr and transformation measured by incorporation of ^{3}H-thymidine.

Discussion

The results of this study demonstrate that: 1) The *in-vitro* culture of sensitized MLNC with egg antigen stimulates MLNC to proliferate and results in the expansion of cells that were responsible for the transfer of protective immunity. 2) There is the continuing proliferation of *H. nana*-specific protective lymphocytes in the mesenteric lymph nodes during the course of *H. nana* infection. These conclusions are supported by the following observations: First, sensitized MLNC well responded specifically to egg antigen, with maximal proliferation observed when antigen was used at a concentration of 200 μg/ml. Second, the protective immunity, as judged by complete rejection of cysticercoids, can not be transferred by using MLNC cultured without egg antigen, whereas transfer of MLNC cultured with egg antigen results in complete rejection of cysticercoids. Third, upon stimulation with egg antigen *in vitro,* MLNC release IL-2 to support the continuing proliferation of sensitized T-cells into the surrounding medium. Fourth, proliferation of MLNC from *H. nana*-infected mice was increased in culture medium containing IL-2, whereas proliferation of such cells in the absence of IL-2 was low level indicating that MLNC from infected mice are very sensitive to IL-2.

The question that arises from the results obtained from proliferation assay is why ^{3}H-TdR incorporation of MLNC prepared from infected mice was higher than that from non-infected mice when MLNC were cultured without egg antigen. In the *H. nana*-mouse system, adult worms attach to the intestinal mucosa for about one month and release a numerous number of eggs into the feces. The eggs, instead of passing from the host, hatch in the intestine where the liberated oncospheres penetrate the intestinal villi and result in internal autoreinfection (Hunninen, 1936), or natural reinfection (Reed *et al.*, 1977). These reports suggest that the resultant reinfection may trigger continuous antigenic stimulation and recognition in the intestine, and in the mesenteric lymph nodes of infected mice. The difference of ^{3}H-TdR incorporation described above may be explained from the reinfection.

We recently observed that BALB/c mice infected with *H. nana* showed delayed type hypersensitivity (DTH) to soluble egg antigen, and intravenous injection with MLNC conferred not only strong protective immunity but also DTH on syngeneic recipient nude mice (Asano *et al.*, unpublished). It is generally accepted that

DTH is divided into four groups; Jones-Mote hypersensitivity; Tuberculin-type hypersensitivity; Contact hypersensitivity, and Granulomatous hypersensitivity. It is also known that sensitized lymphocytes prepared from DTH, with the exception of Jones-Mote hypersensitivity, positive mice can release MIF upon the *in-vitro* stimulation with specific antigens. Since the results in Table 3 show that MLNC from infected mice release high levels of MAF but not MIF, it may be, therefore, suggested that the oral inoculation with eggs of *H. nana* initiate Jones-Mote hypersensitivity into mice. Further experiments are needed to clarify this point.

A marked inflammatory reaction with predominant infiltration of neutrophils, eosinophils and monocytes was reported in the intestine of mice infected with *H. nana* (Miyazato *et al.*, 1979, Friedberg *et al.*, 1979). These results may suggest that the lymphocytes generated by *H. nana* infection are able to release mediators affecting neutrophils and eosinophils when the mice were orally challenged with eggs. Further analysis of lymphokine production by MLNC of *H. nana*-infected mice lead to more precise understanding of the mechanisms underlying the cysticercoid rejection from the intestinal villi.

Acknowledgment

We gratefully acknowledge the skilled technical assistance of Ms. M. Shinoda.

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