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Adherence Reactions with Mouse Lymphoid Cells against the Oncosphere Larvae of *Hymenolepis nana*.

TADAAKI FURUKAWA

Department of Medical Biology, School of Medicine, Showa University, Tokyo, Japan

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Introduction

Several types of immune responses have been clearly shown in mice during infection with *Hymenolepis nana*, including a strong protective immunity against reinfection (Hunnienen, 1935 ; Hearin, 1941 ; Heyneman, 1962, a, b) and serum antibodies with a wide variety of immunological activities (Larsh, 1943 ; Weinmann, 1966 ; Coleman and De Sa, 1964 ; Coleman *et al.*, 1967, 1968 ; Di Conza, 1969). In *H. nana* infection, the host is exposed to three different stages of helminth's life cycle ; oncospheres, cysticercoids in tissue phase and adult worms in lumenal phase. Each of these presumably presents a variety of stage specific, as well as shared, antigens, thus contributing to the complexity of identifing any particular antigen which is capable of inducing protective immunity in infected mice. Recently Ito (personal communications) has demonstrated the agglutination of oncosphere larvae of this tapeworm in the immune serum of mice. Though the exact role of serum antibodies in the protective immunity to *H. nana* infection is still controvertial, the observation by Ito has fortified the hypothesis that the larval stage of *H. nana* has an important role in producing functional antigens (Weinmann, 1966 ; Di Conza, 1969). It was also suggested that living oncosphere larvae would be an useful test antigen for studying immune mechanisms in this host-parasite system. A wide variety of living parasites, usually the larval forms, have been used in *in vitro* system to investigate various effects of the immune response on parasites (Soulsby, 1967).

In addition to some reports on the serological responses to *H. nana* in mice, a number of evidences recently reported by Okamoto (1968, 1970) and Okamoto and Kondo (1972) seems to support the view that the acquired resistance to reinfection in mice is, at least partly, attributable to a cell mediated immunity. This leads to debates whether the mechanism involved is primarily humoral or cellular. However, the sequence of events in cellular responses to *H. nana* is less understood than in humoral responses, chiefly due to the lack of appropriate assay methods.

Preliminary observations in our laboratory using artificially hatched oncosphere larvae of *H. nana* have shown that some lymphoid cells obtained from mice infected with *H. nana* aggregated on or around the larvae *in vitro*. Subsequently the present author attempted to study the cell adherence reaction with cells from spleens, Peyer's patches and mesenteric lymph nodes of mice against *H. nana* larvae with or without cooperation of homologous immune serum. The latter two gut-associated lymphoid tissues were chosen with the hope of obtaining some informations concerning localized immune responses which have been known to be one of the characteristic features accompanied with *H. nana* infection in mice (Heyneman, 1962a ; Weinmann, 1966). Evidence will be presented in the present report that the cell adherence reaction is a manifestation of immune responses which develop as a consequence of *H. nana* in-

fection in mice, and that humoral antibody has an important role in inducing cellular reactions against *H. nana* larvae.

Materials and Methods

Animals and infection : Inbred strain of Swiss albino mice were derived from our own colony maintained by strict brother and sister mating. At 6 weeks after birth mice were infected orally with 2000 *H. nana* eggs. The egg-shells were removed by stirring them with glass beads (Berntzen and Voge, 1965). Six mice randomly chosen from this group 4 days after infection had an average of 752 intestinal cysticercoids (range, 509–973). Examination for cysticercoids in the intestinal villi followed the method of Okamoto (1968). A number of mice maintained carefully in worm-free environment served as controls. In order to ascertain that these mice were free from accidental contamination with *H. nana*, feces were examined at weekly interval by brine floatation method.

In vitro hatching of eggs : H. nana eggs were obtained from gravid proglottids of adult worms, washed with physiological saline and stored at 4°C. The *in vitro* hatching of eggs was performed according to the method of Berntzen and Voge (1965) with a minor modification in which Hank's balanced salt solution was used, instead of Tyrode's, as a basal medium of hatching solution. Oncosphere larvae were washed three times with sterile Hank's solution by gentle centrifugation. Finally they were suspended in Eagle's minimum essential medium supplemented with normal mouse serum (5%, v/v) which had been inactivated at 56°C for 30 minutes. The final medium will be referred to as MEM+NMS (pH 7.2). All the experiments were conducted within 2 hours after hatching.

Preparation of cell suspensions : Spleens, Peyer's patches and mesenteric lymph nodes were removed aseptically from bled out mice. Cell suspensions were prepared by gently teasing respective tissues in cold Hank's solution. The cell suspensions were passed through two layers of fine nylon cloth to remove gross tissue debris and clotted cells and was washed twice with Hank's solution at 4°C. Finally cells were suspended in MEM+NMS and adjusted to contain 5×10^6 nucleated cells/ml. Cell counts were performed with haemocytometer and viability was determined by trypan blue exclusion. In each experiment, one mouse was usually killed and spleen cells were prepared from it, while 3 to 4 mice were required for obtaining a sufficient number of Peyer's patch and mesenteric lymph node cells. Cells obtained from mice infected with *H. nana* eggs will be referred to as sensitized cells, and those obtained from uninfected mice as normal cells.

Mouse sera, rabbit anti-mouse globulin serum and chemicals : Both uninfected and infected mice were exsanguinated by cardiac puncture. Sera were separated, inactivated at 56°C for 30 minutes and stored at −30°C. Sera obtained from infected mice will be referred to as immune sera and those from uninfected mice as normal sera.

The rabbit anti-mouse globulin serum was prepared as follows ; normal mouse sera were fractionated by precipitation with 30% ammonium sulphate in 0.2 M tris-HCl buffer (pH 7.5) at 4°C. The resulting precipitate was dissolved, dialysed against physiological saline and suspended in Freund's complete adjubant. This preparation was injected repeatedly into the food pats of a rabbit. The rabbit anti-mouse globulin serum thus produced was inactivated at 56°C for 30 minutes. Normal rabbit serum was also heat-inactivated and served as control. Commercially available rabbit anti-mouse globulin serum (Miles laboratory) was used in some experiments.

Iodoacetate and puromycin dihydrochloride (Nutritional Biochemicals) were dissolved in MEM at 10^{-3} to 10^{-4} M and pH was adjusted to 7.2 with 0.1 N NaOH.

Experimental procedures : Aliquots of oncosphere suspension and cell suspension were poured into a Wasserman test tube so that 2.5×10^6 cells and 5×10^3 larvae may

come into contact in a final volume of 1 ml MEM+NMS. This gave a cell-to-larva ratio of about 500 : 1. Unless otherwise stated, the mixtures were incubated at 37°C for 2 hours with gentle agitation every 30 minutes. After incubation, mixtures were suspended gently but throughly with a pippet and a drop of medium was transferred on a slide glass. A total of 500 to 1000 larvae for each tube was assayed under the microscope and the number of cells attached firmly to the surface of the larvae was counted irrespective of cell types. The larvae were designated as those with more than six adhering cells, less than six cells, or no adhering cells. The results were expressed as the percentage of the larvae having more than 6 adhering cells to facilitate comparison of the values obtained (see Results). In some experiments, culture medium was supplemented with sera from infected mice or reagents tested at various concentrations. Details will be described in Results.

Representative samples of the larvae with adherent cells were fixed in a mixture of ethanol, methanol and acetone (2 : 1 : 1) and stained with methyl-green and pyronin (Morseth and Soulsby, 1969 a).

Results

Morphological observations.

Representative examples of the adherence of spleen cells against *H. nana* larvae after 2 hours of incubation at 37°C are shown in Photo. 1–5. The spleen cells appeared to adhere to the larval surfaces regardless of previous *H. nana* infection in mice. However, the larvae in normal spleen cell suspensions tended to have fewer adherent cells (Photo. 1). In contrast, spleen cells obtained from mice 3 weeks after infection exhibited a more adhesive behavior, sometimes forming clumps consisting of 10 or more cells (Photo. 2–5). The sensitized cells were randomly adherent to the entire surface of the larvae (Photo. 2–4), but some larvae exhibited a polar adherence of cells (Photo. 5). All these photographs show that adhering cells did not exert any adverse effect on the morphology of the larvae. A similar adherence reaction was observed after 6 and 18 hours of incubation at 37°C. The degree of the cell adherence reaction with Peyer's patch and mesenteric lymph node cells obtained from mice 3 weeks after infection was quite low. The formation of large cell aggregates was seldom observed. An example of the adherence of Peyer's patch cells from infected mice is shown in Photo. 6. General morphology of the larvae incubated in respective cell suspensions at 37°C for up to 18 hours showed no particular differences as compared with the larvae incubated in medium alone.

Spherical form of the larvae and the formation of large cell aggregates made it difficult to classify the individual spleen cells adhering to the larvae. However, it was noted that adhering cells comprised a very heterologous populations. A limited observation with preparations stained with methyl-green and pyronin suggested that adhering cells were mainly consisted of small and medium size mononuclear cells with a purply or darkly stained nucleus and limited amounts of cytoplasm. The cytoplasm of these cells showed little or no pyronin staining. Polymorphonuclear leucocytes were not contained in the cell aggregates.

Quantitative studies of the spleen cell adherence reaction.

Comparative studies were made on the number of normal and sensitized spleen cells adhering to the larvae to provide a basis for discriminating between random and specific attachment of cells. Spleen cells from uninfected mice and those from mice 3 weeks after infection were incubated with the larvae at 37°C for 2 hours at a cell-to-larva ratio of 500 : 1. Table 1 shows the result of five sets of the experiment, in which the distribution of larvae among populations of 500 to 800 larvae with 1 to >10 adhering cells is recorded. The number of larvae having adherent cells was greater in sensitized cell suspensions than in normal cell suspensions. The difference of both groups

Table 1 Range of mouse spleen cells attached to the oncosphere larvae of *H. nana* incubated with normal and sensitized cells at 37°C for 2 hours at a cell-to-larva ratio of 500 : 1.

Spleen cell[1]	Total number of larvae assayed	Number of larvae with following adhering cells				% larvae with ≥6 adhering cells	
		Number of cells					
		0	1–5	6–10	>10		
Normal	529	417	110	1	1	0.4	Mean±SE[2]
	662	594	67	1	0	0.2	
	580	557	22	0	1	0.2	
	747	678	64	3	2	0.7	0.4±0.1
	600	514	82	4	0	0.7	
Sensitized	593	445	82	31	35	11.1	
	526	354	93	37	42	15.0	Mean±SE
	830	543	191	51	43	11.3	
	580	410	85	21	64	14.7	13.5±0.9
	618	402	122	38	56	15.2	

1. Normal spleen cells were obtained from uninfected mice and sensitized cells from mice 3 weeks after an oral infection with 2000 shell-removed eggs of *H. nana*.
2. Standard error of the mean.

was particularly apparent when the percentage of the larvae with more than six adhering cells was compared. Only the combination of the sensitized cells and the larvae gave an appreciable increase in the percentage of such larvae. The result confirmed the above observations that spleens from infected mice contain specific cell populations which are capable of forming large cell aggregates on or around the surface of the larvae.

Effects of the ratio of cell-to-larva, incubation time and temperature on the spleen cell adherence reaction are illustrated in Table 2 and 3. Spleen cells were derived from uninfected mice and from mice 3 weeks after infection. Higher concentration of normal spleen cells led to an increase in non-specific attachment during 2 hours of incubation at 37°C, whereas the dilution of sensitized cells greater than 250 : 1 markedly reduced the incidence of the adherence reaction and consequently diminished the sensitivity of the experiment (Table 2). Optimal adherence of sensitized spleen cells occurred after incubation of mixtures at 37°C for 2 hours at a cell-to-larva ratio of 500 : 1 (Table 3). There was less adherence of cells in mixtures incubated at 4°C for 2 hours. Optimal ad-

Table 2 Effect of cell-to-larva ratio on the cell adherence reaction to *H. nana* larvae with normal and sensitized spleen cells. All the experiment were carried out at 37°C for 2 hours.

Ratio of cell-to-larva	% larvae with ≥6 adhering cells[1]	
	Spleen cells[2]	
	Normal	Sensitized
125 : 1	0.0	1.2
250 : 1	0.0	2.5
500 : 1	0.7	12.7
1000 : 1	1.4	14.8
2000 : 1	2.7	22.1

1. Arithmetic means of 4 experiments.
2. Normal spleen cells were obtained from uninfected mice and sensitized cells from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana*

Table 3 Effect of incubation temperature and time on the cell adherence reaction to *H. nana* larvae with normal and sensitized spleen cells. All the experiments were carried out at a cell-to-larva ratio of 500 : 1.

Temperature (°C)	Time (Hour)	% larvae with ≥6 adhering cells[1]	
		Spleen cells[2] Normal	Sensitized
37	2	0.4	12.9
	6	1.7	12.1
4	2	0.9	3.7
	6	1.0	16.7

1. Arithmetic means of 5 experiments.
2. Normal spleen cells were obtained from uninfected mice and sensitized cells from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana*.

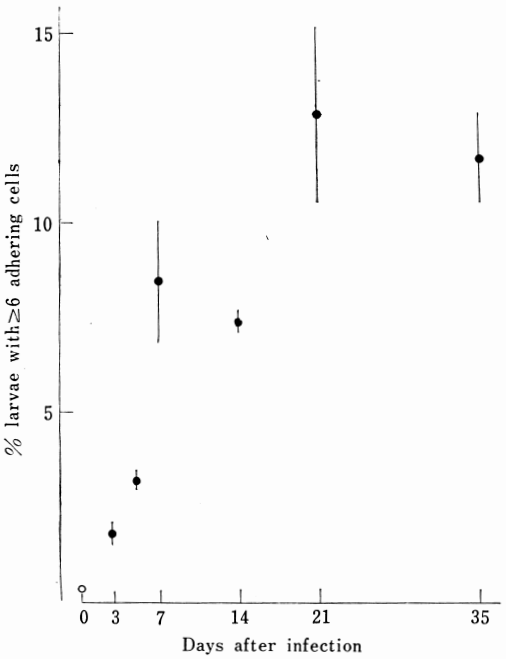

Fig. 1 The appearance of the cell adherence reaction with spleen cells of mice following infection with 2000 shell-removed eggs of *H. nana*. The vertical bars represent standard error of the mean.

herence, likewise, occurred after 6 hours of incubation at 4°C. Accordingly, the incubation of cells and larvae at 37°C for 2 hours at a cell-to-larva ratio of 500 : 1 was selected for routine use in the following experiments. The results obtained were arbitrarily compared with regard to the percentage of the larvae having more than six adhering cells.

The adherence reaction with spleen cells obtained from twelve uninfected mice and from groups of 5 to 6 mice at a time ranging from 3 to 35 days after infection was examined to determine the kinetics of the appearance of this reaction following infection. Results are shown in Fig. 1. A significant cell adherence reaction clearly emerged 3 days after infection, thereafter increasing rapidly and reaching to a peak at the 7th day. A gradual increment was observed up to 35 days after infection.

Influence of rabbit anti-mouse globulin serum and metabolic inhibitors on the spleen cell adherence reaction was studied in order to determine whether the phenomenon observed depends upon a specific immunological reaction. Spleen cells obtained from mice 3 weeks after infection were suspended in MEM and incubated for 30 minutes at 37°C with an equal volume of rabbit anti-mouse globulin serum, 10^{-4}M iodoacetate or 10^{-3}M puromycin. After incubation, the larvae were directly added in the cell suspension and the adherence test was carried out at 37°C for 2 hours. Controls were set up in MEM+NMS and in the medium containing an equal volume of normal rabbit serum. The result is given in Table 4. It was shown that all the reagents tested were powerful inhibitors of the cell adherence reaction, resulting in a reduction to less than 30% of the control level.

Adherence reactions with Peyer's patch and mesenteric lymph node cells.

The reactivity of Peyer's patch and mesenteric lymph node cells against the larvae was examined under the same experimental conditions and criterion as described in the previous experiments using spleen cells.

Table 4 Effect of rabbit anti-mouse globulin serum, iodoacetate and puromycin on the cell adherence reaction to *H. nana* larvae with sensitized spleen cells.

Treatment of cells[1]	% larvae with ≥6 adhering cells±SE[2]	% reduction
Rabbit antiserum	3.2±0.7	74.2
Normal rabbit serum	13.8±2.2	Nil
MEM+NMS (Control)	12.4±1.3	–
Iodoacetate	1.0±0.5	92.3
Puromycin	1.8±0.5	86.2
MEM+NMS (Control)	13.0±1.6	–

1. Spleen cells obtained from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana* were incubated with 25% rabbit antiserum, 10^{-4} M iodoacetate or 10^{-3} M puromycin at 37°C for 30 minutes before carrying out the adherence test.
2. Arithmetic means of 5 experiments±standard error of the mean.

Each of the tissues were obtained from mice 3 weeks after infection. Results of the adherence test are summarized in Table 5. The degree of the adherence of Peyer's patch cells to the larvae was quite low. There was no difference between cells from infected mice and those from uninfected mice in adhering to the larvae. A slightly enhanced reaction was observed in 3 out of 5 experiments with mesenteric lymph node cells from infected mice as compared with controls. However,

Table 5 Cell adherence reaction with mouse Peyer's patch and mesenteric lymph node cells incubated with *H. nana* larvae at 37°C for 2 hours at a cell-to-larva ratio of 500 : 1.

Tissue	Cell[1]	Number of determination	% larvae with ≥6 adhering cells±SE
Peyer's patch	Normal	7	0.2±0.1
	Sensitized	7	0.2±0.1
Mesenteric lymph node	Normal	5	0.2±0.1
	Sensitized	5	2.1±0.9

1. Normal cells were obtained from uninfected mice and sensitized cells from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana*

the values obtained was variable, ranging from 2.2 to 5.1%. In any case, the reactivity of mesenteric lymph node cells was not comparable to that seen with the spleen cells from infected mice.

Influence of immune serum on the reactivity of spleen and Peyer's patch cells.

The assay system described above was employed to determine the influence of immune sera on the reactivity of lymphoid cells against *H. nana* larvae. Spleen cells, Peyer's patch cells and sera were obtained from mice 3 weeks after infection and, as for controls, from uninfected mice. The following experiments consisted of four combinations of cells and sera. The larvae were incubated with either normal or sensitized cells in MEM supplemented with either

Table 6 Effect of immune serum on the cell adherence reaction to *H. nana* larvae with normal and sensitized spleen cells.

Cell[1]	Serum[1]	% larvae with ≥6 adhering cells	Mean±SE
Normal	Normal	0.4, 0.8, 1.0, 1.1	0.8±0.2
	Immune	50.6, 54.8, 56.8, 68.5, 70.6	60.3±3.9
Sensitized	Normal	16.3, 20.2, 25.0, 27.2	22.2±2.4
	Immune	24.1, 65.5, 70.6, 70.6, 71.1	60.5±9.2

1. Normal cells and sera were obtained from uninfected mice. Sensitized cells and immune sera were obtained from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana*. The concentration of serum in each experiment was 25%.

Table 7 Effect of immune serum on the cell adherence reaction to *H. nana* larvae with normal and sensitized Peyer's patch cells.

Cell[1]	Serum[1]	% larvae with ≥6 adhering cells	Mean±SE
Normal	Normal	0.0, 0.0, 0.0, 0.0	0.0
	Immune	17.0, 38.3, 52.3, 53.3, 75.8	47.3± 9.7
Sensitized	Normal	0.0, 0.0, 0.0, 0.0	0.0
	Immune	10.0, 23.0, 32.7, 42.0, 47.3, 83.4	39.7±11.5

1. Normal cells and sera were obtained from uninfected mice. Sensitized cells and immune sera were obtained from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana*. The concentration of serum in each experiment was 25%.

normal or immune serum at a final concentration of 25% (v/v). The number of larvae with more than 6 adhering cells was counted at the end of a 2 hour incubation period at 37°C. Results are indicated in Table 6 and 7. Spleen cells derived from uninfected and infected mice could adhere to the larvae in the presence of immune serum (Table 6). All the immune sera tested had a wide range of activity and, thus, the individual values obtained showed a considerable variance. In general, the degree of the cell adherence reaction in the presence of immune serum was similar with regard to the normal and sensitized spleen cells. A similar result was obtained with Peyer's patch cells (Table 7). As indicated in Photo. 7 and 8, the cell adherence reaction with immune serum was more intense than that seen with sensitized spleen cells in the absence of immune serum (Photo. 2–5). Though the exact identification of the various cells adhering to the larvae was difficult, observation of several specimens revealed that there was a mixed population of cells. An apparent agglutination of the larvae was observed in mixtures containing immune serum. Photo. 9 and 10 show that these larvae also had a number of adhering cells on their free surfaces.

The mode of action of immune serum was further studied by the following experiment. Normal spleen cells had been incubated with immune serum at a concentration of 25% for 30 minutes at 37°C, washed throughly with MEM and then tested for adherence against untreated larvae, whereas the larvae pretreated similarly with immune serum were subsequently mixed with untreated normal spleen cells. Both tests were performed in MEM+NMS at 37°C for 2 hours. Results are summarized in Table 8. Normal spleen cells could adhere to the larvae which had been pretreated with immune serum. There was, however, little or no adherence of normal spleen cells pretreated with immune serum to untreated larvae.

Table 8 Effect of pretreatment of cells or larvae with immune serum on the cell adherence reaction to *H. nana* larvae with normal spleen cells.

Reaction mixture[1]	% larvae with ≥6 adhering cells ± SE[2]
Cells+Treated larvae	45.8±9.5
Treated cells+Larvae	1.4±0.6
Cells+Larvae (Control)[3]	0.7±0.2

1. Normal spleen cells or larvae were pre-incubated with immune serum at a concentration of 25% and washed with MEM before carrying out the adherence test.
2. Arithmetic means of 5 experiments±Standard error of the mern
3. Controls for normal spleen cells and untreated larvae.

Factors inducing the cell adherence reactions were determined by absorbing immune sera with rabbit anti-mouse globulin serum. Sera obtained from mice 3 weeks after infection were mixed with four volumes of rabbit anti-mouse globulin serum and left overnight at 4°C. Mixtures were centrifuged and supernatants were tested for induction

Table 9 Effect of absorption of immune sera by rabbit anti-mouse globulin serum on the serum-mediated cell adherence reaction to *H. nana* larvae with normal spleen cells.

Treatment of immune serum[1]	% larvae with ≥6 adhering cells±SE[2]
Rabbit antiserum	1.5±0.5
Normal rabbit serum	25.9±5.1
Control[3]	1.6±0.5

1. Sera obtained from mice 3 weeks after infection with 2000 shell-removed eggs of *H. nana* were treated with four volumes of rabbit antiserum.
2. Arithmetic means of 6 experiments±Standard error of the mean.
3. Controls for normal spleen cells in MEM+NMS.

of the cell adherence reaction. Immune sera treated with the same amount of normal rabbit serum served as controls. The adherence test was done at 37°C for 2 hours in mixtures of normal spleen cells, larvae, and treated sera at a concentration of 50%. Thus, the final concentration of mouse immune serum in each tube was 10%. As indicated in Table 9, a significant reduction in the cell adherence reaction was observed after absorption of immune sera by rabbit anti-mouse globulin serum.

Discussion

The results reported in this paper indicated that the spleen of mice infected with *H. nana* eggs involved cell populations which had an ability to adhere to the surface of artificially hatched oncosphere larvae of this tapeworm. The adherence of sensitized spleen cells could be detected within 2 hours of incubation at 37°C without adding homologous immune serum *in vitro*. There is at present no suitable method for the quantitation of cell adherence reactions against parasitic worms. Higashi and Chowdhury (1970) and Hudson *et al.* (1971) have described a quantitative parameter which involves cell counts on parasites. This method was not readily applicable in the present study because of the formation of closely packed cell aggregates and the variability of the reactions encountered. The quantitation of the cell adherence reaction was further complicated by the presence of normal spleen cells which caused a non-specific attachment against the larvae. However, the analysis of the data obtained under the definitive experimental conditions suggested that the difference between normal and sensitized spleen cells in adhering to *H. nana* larvae can be quantitatively representative by comparing the number of larvae with more than six adhering cells. Increase in the number of such larvae can be regarded as reflecting the increase in the number of reactive cells in the spleen of infected mice. A similar attempt to establish specific method for quantitation of the cell adherence reactions to parasitic worms has recently been reported by Stankiewicz and Jeska (1973). In the present study, it was difficult to assess with any certainty the exact types of cells in contact with the larvae. However, a limited observation suggested that the usual cell aggregates comprised a mixed cell populations in which small and medium size mononuclear cell were predominant.

The spleen cell adherence reaction was detectable from the third day of infection, reaching a peak shortly afterwards and remaining elevated throughout the study. Consequently, it appears that reactive cells in the spleen had developed in response to *H. nana* infection in mice. Ito (personal communications) has shown that serum antibody anainst *H. nana* larvae could be demonstrable from the second week of infection. The appearance of reactive cells in the spleen of infected mice could be detected long before the appearance of serum antibody. These findings are convincing evidences that parenteral immune responses develop following *H. nana* infection in mice. Additional evidence has been reported by Di Conza (1970) and Furukawa (1971) who showed that the development of the larvae in the ectopic sites of mice was entirely

inhibited when shell-removed eggs were injected into infected mice.

There is a discrepancy between the peak response of spleen cells and the establishment of protection against challenge infection, since Heyneman (1962 b) has reported that a nearly maximum level of protection manifested as early as 2 days after initial infection. This fact suggests that resistance to *H. nana* infection can be explained on the basis of immune mechanisms operating locally in the intestinal tract. Thus, cell populations reactive to *H. nana* larvae in Peyer's patches and mesenteric lymph nodes of mice were studied to determine whether reactive cells in areas of local response to infection are extensive. The present study showed that these gut-associated lymphoid tissues of infected mice contained none or only occasional cells which were reactive to the larvae. Though possibility remains that such cells are involved in these lymphoid tissues at a rate low enough to be missed by the present experiment because of its sensitivity limits, the present study failed to detect any particular responses localized to the lymphoid tissues draining infection sites of *H. nana* larvae.

The lymphoid cell adherence reactions against parasitic worms have been noted by several workers and shown to be dependent, at least in part, upon antibody (Newsome, 1962; Crandall *et al.*, 1967; Soulsby, 1963, 1967; Morseth and Soulsby, 1969 a, b; Jeska, 1969; Higashi and Chowdhury, 1970; Stankiewicz and Jeska, 1973). Since the adherence of sensitized spleen cells to *H. nana* larvae occurred without adding immune serum, it is important to determine whether the cells adherent to the larvae are committed to humoral antibody production or to cell mediated immune reactions. The approach taken to this question was to apply relevant reagents such as rabbit anti-mouse globulin serum, an antimetabolite (iodoacetate) or an inhibitor of protein synthesis (puromycin) to the sensitized spleen cells. It was shown that the adherence of sensitized spleen cells to the larvae was blocked by the addition of these reagents into culture medium. Moreover, the appearance of the optimal cell adherence reaction was delayed at 4°C as compared with 37°C. These results are in agreement with that of others with different experimental systems. Moav and Harris (1970) have shown that the clustering of heterologous red blood cells against sensitized lymphoid cells (rosette formation) was inhibited by several antimetabolites and inhibitors of protein synthesis. Helmreich *et al.* (1961) have reported that secretion of antibodies by isolated cells was inhibited at 4°C. These findings suggest the possibility that the adherence of spleen cells to *H. nana* larvae is related to the active synthesis of antibody by isolated cells in culture, and attest to the involvement of antibody in the reaction. No evidence has yet been found to indicate unequivocally that the spleen cell adherence reaction is an expression of cell mediated immune reactions.

A most interesting finding is the fact that serum from infected mice had an ability to induce strong cell adherence reactions against *H. nana* larvae. The degree of the cell adherence reaction induced by immune serum seemed to be similar with regard to spleen and Peyer's patch cells obtained from normal and infected mice. However, the individual values showed a degree of variance, probably reflecting the immune status of sera used in the experiment. It is interesting to note that Peper's patch cells could adhere to the larvae in the presence of immune serum, while these cells were considered to be essentially non-reactive in the absence of immune serum. It may be concluded therefore that the serum-induced cell adherence reaction is primarily dependent on factors involved in the immune serum. These factors have several properties characteristic of antibody, since (a) they were heat-stable (56°C, 30 minutes); (b) they were exclusively present in sera obtained from infected mice; and (c) they could be absorbed with rabbit anti-mouse globulin serum and thus be removed from immune sera. In view of the intensity of the cell adherence reactions in the presence of immune serum, the present results suggest that humoral antibodies may have a primary

importance in inducing subsequent cellular responses.

Further observations indicated that normal spleen cells pre-incubated with immune serum and washed before carrying out the adherence test did not give rise to the reaction. In contrast, normal spleen cells could adhere to the larvae which had been pre-incubated with immune serum. Thus, it is likely that the cell adherence reaction in this case was not due to a cytophilic antibody but due to an antigen-antibody complex presumably formed on the surface of the larvae.

It has been observed that the antibody-mediated cell adherence reaction occurs with several species of parasitic worms. However, there is no agreement ragarding the cell types involved in the reaction. Thus, macrophages, neutrophils, eosinophils or pyroninophils have been found to adhere to a number of parasites *in vitro* (Newsome, 1962 ; Soulsby, 1963, 1967 ; Morseth and Soulsby, 1969 a, b ; Higashi and Chowdhury, 1970). Lymphoid cells used in the previous studies cited above were derived from peripheral blood or peritoneal exudates of patients or previously sensitized animals. The present study clearly shows that cells obtained from spleens and at least one of the lymph nodes draining the infection sites of *H. nana* are involved in the antibody-mediated cell adherence reaction.

Despite the marked reactivity of several lymphoid cells in adhering to *H. nana* larvae, especially in the presence of immune serum, the exact type of adhering cells and the nature of the function of these cells remain unknown. Although some descriptive accounts of histopathology are available (Bailey, 1951 ; Weinmann and Lee, 1964 ; Di Conza, 1970 ; Furukawa, 1971), relatively little is known concerning the mechanisms of direct cellular actions against *H. nana* larvae. In the observations reported in this paper, the adhessiveness of cells and general morphology of the larvae were examined only after a short-term incubation. Subsequent experiments involving longer periods of incubation will help to determine if the death or inhibition of the development of the larvae become more extensive.

It is important to bear in mind that there may be a highly dynamic process *in vivo*, in which fully functional lymphoid cells and antibody are habitually migrated to the parasitized tissues. From this point of view, the apparent role of immune serum on the cell adherence reaction may be significant because it can provide means for preferential recruitment and incorporation of not only specific reactive cells but also of non-reactive cells against challenge larvae. These concepts lend weight to the assumption, though still highly speculative, that anti-parasitic activities of all the known humoral and cellular factors, and possibly others yet unknown, would be superimposed in foci of reinfection and, thus, *H. nana* larvae would be affected in more than one way in the intestinal villi of previously infected mice.

Summary

A series of *in vitro* studies were undertaken to determine the possible influence of lymphoid cells of mice infected with *Hymenolepis nana* on the larval stage of this tapeworm. Artificially hatched oncosphere larvae of *H. nana* were incubated with lymphoid cells obtained from spleens, Peyer's patches and mesenteric lymph nodes of uninfected and infected mice at 37°C for several hours *in vitro*. Microscopic observations up to 18 hours of incubation did not indicate any particular changes on the general morphology of the larvae. Spleen cells from infected mice caused a marked adherence reaction against the larvae within 2 hours of incubation at 37°C. The spleen cell adherence reaction was detectable from the third day of infection. This reaction was blocked by the rabbit anti-mouse globulin serum, iodoacetate and puromycin, and was delayed at 4°C as compared with 37°C. It was suggested that the active synthesis of antibody by isolated cells was related to this phenomenon. Peyer's patch and mesenteric lymph node cells obtained from infected

mice did not give rise to the detectable adherence reaction. Immune sera had an enhancing effect on the adherence reaction with spleen and Peyer's patch cells obtained from uninfected and infected mice. Normal spleen cells could adhere to the larvae preincubated with immune sera, whereas normal spleen cells preincubated with immune sera did not adhere to untreated larvae. Absorption of immune sera by rabbit anti-mouse globulin serum abolished the enhancing effect. It was concluded that factors, possibly of antibody nature, could combine with the antigenic sites of the larvae and mediate the cell adherence reaction. The significance of the cooperation between humoral and cellular factors in the immune response to *H. nana* infection in mice was emphasized. The exact types of cells adhering to the larvae and the mode of action of these cells upon the larvae remain unknown.

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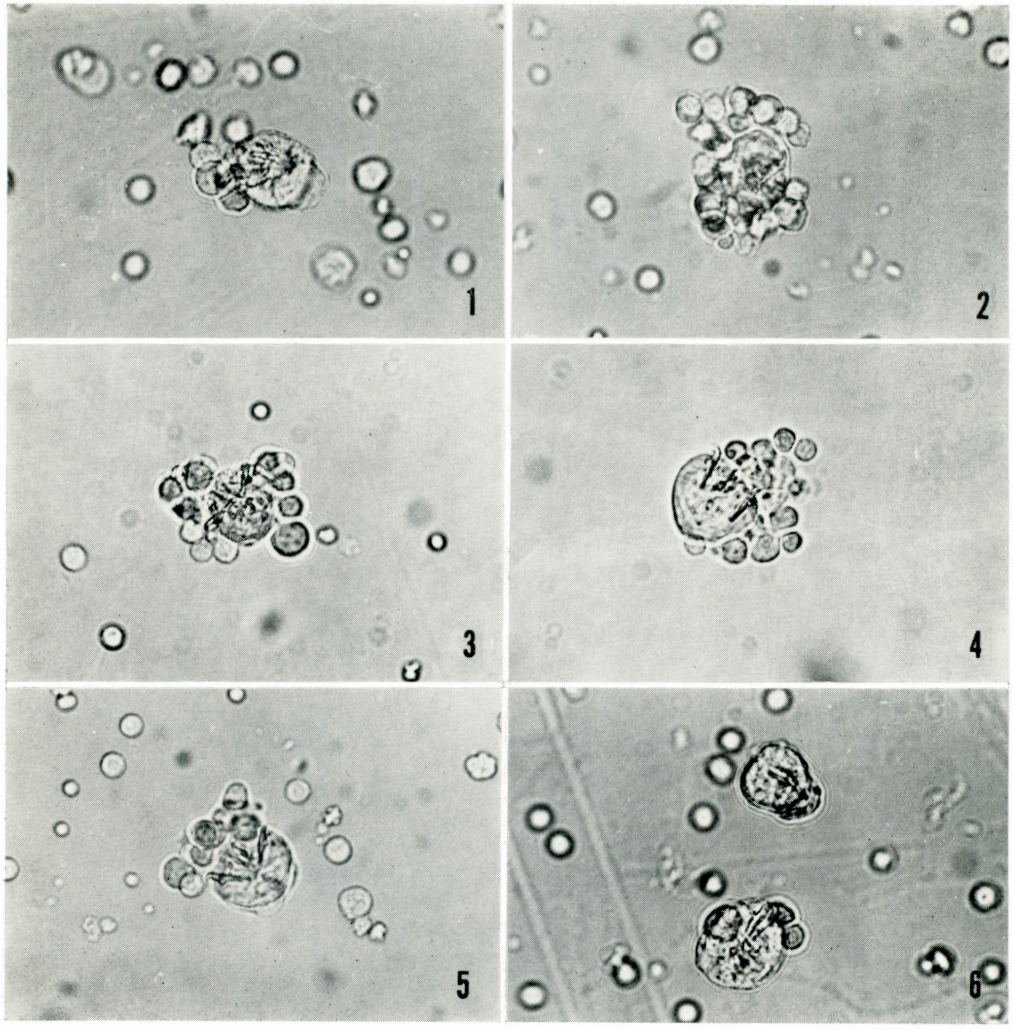
小形条虫六鉤幼虫に対するマウスのリンパ系細胞の付着反応

古 川 忠 明
（昭和大学医学部医動物学教室）

人工孵化法により得た小形条虫の六鉤幼虫と，虫卵感染マウスの脾細胞を混合し，最高18時間インキュベートして幼虫に対するリンパ系細胞の影響を観察した．感染マウスの細胞とともにインキュベートした幼虫は，未感染マウスの細胞とともに，あるいは培養液単独でインキュベートした幼虫と比べて形態学的に差異を認めなかつた．この間に，幼虫に対する細胞の付着反応が認められ，その検索方法について詳述した．脾細胞の付着反応は，虫卵感染3日後から検出可能であつた．この反応は，マウス血清グロブリンに対するウサギ抗血清．ヨード酢酸，ピュロマイシンによつて抑制され，また4°Cでは37°Cに比べて反応が遅延した．虫卵感染マウスのパイエル板細胞および腸管膜リンパ節細胞の付着反応は，脾細胞のそれに比べて極めて低かつた．マウスの抗血清を培養液中に加えると，顕著な細胞付着反応が観察された．その反応の程度は，脾細胞あるいはパイエル板細胞のいずれを用いても，また各々の細胞が，未感染あるいは感染マウスのいずれに由来するものであつてもほぼ同様であつた．未感染マウスの脾細胞は，あらかじめマウス抗血清で処理し洗浄した幼虫に対して付着反応を示した．しかし，抗血清で前処理した脾細胞は，未処理の幼虫に対する付着反応を示さなかつた．マウスの抗血清を前述のウサギ抗血清で吸収すると，細胞付着反応に対する効果が失なわれた．



Explanation of Photographs

All the figures were photographed in wet mounts after incubation of mouse lymphoid cells and living oncosphere larvae of *H. nana* at 37°C for 2 hours *in vitro*. Light microscope, × 550.

Photo. 1. Oncospher larva of *H. nana* incubated with normal spleen cells. Three adhering cells are seen on the larval surface.

Photo. 2-5. Cell adherence reaction to *H. nana* larvae with spleen cells obtained from mice 3 weeks after infection, showing variable number of cells on the larvae. Photo. 5 shows polar adherence of cells.

Photo. 6. *H. nana* larvae incubated with Peyer's patch cells obtained from mice 3 weeks after infection.

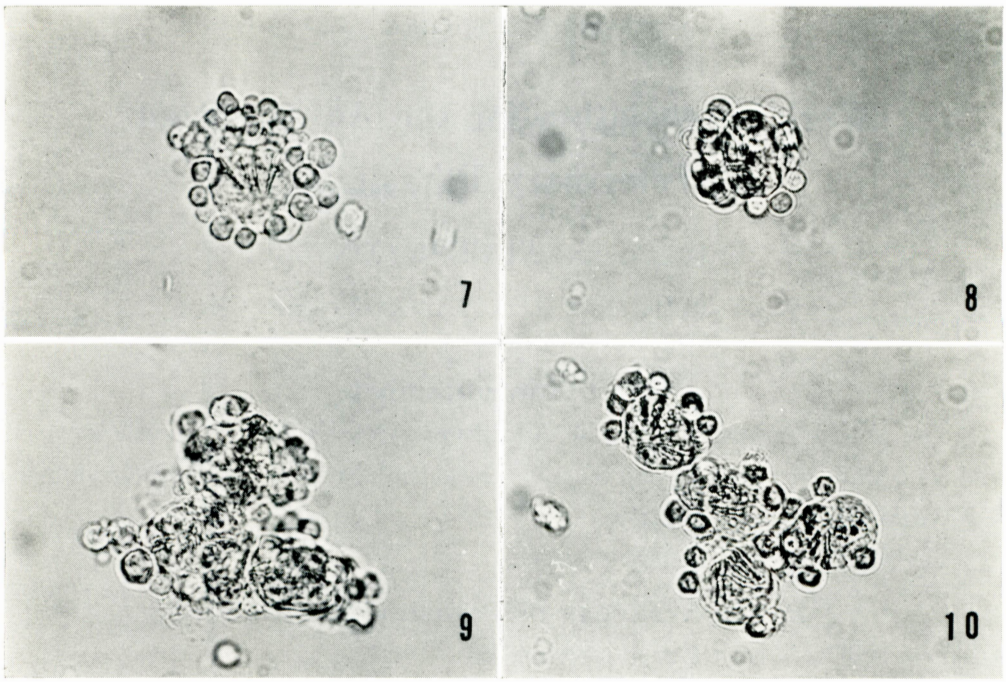


Photo. 7. Adherence of normal spleen cells to *H. nana* larvae in the presence of immune serum.

Photo. 8. Adherence of normal Peyer's patch cells to *H. nana* larvae in the presence of immune serum.

Photo. 9. Adherence of normal spleen cells to *H. nana* larvae agglutinated in immune serum.

Photo. 10. Adherence of normal Peyer's patch cells to *H. nana* larvae agglutinated in immune serum.