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Th2 Response and Granuloma Formation to Eggs of *Schistosoma japonicum* in Mice

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Abstract: BALB/c, C57BL/6, C3H/He and ddY mice were injected i.v. with 10,000 eggs of *Schistosoma japonicum* to induce pulmonary granuloma formation. All mice were sacrificed two weeks later. The size of granulomas in the lungs and the cytokines secreted from the spleen cells of the four strains of mice were compared. IL-3, IL-4 and IL-6 were detected in the supernatants from 3-day cultured spleen cells in all strains of mice. The spleen cells restimulated with the soluble egg antigen of *S. japonicum* produced significantly higher levels of the above three cytokines than unstimulated cells. C3H/He was the highest, while BALB/c was the lowest responder to the eggs. IL-2, IFN-γ and TNF-α were undetectable in all strains of mice. The size of pulmonary granulomas was similar among the four strains. This suggested that the Th2 (T helper type 2) response could be induced to the eggs of *S. japonicum* in the four strains of mice.

Key words: *Schistosoma japonicum*, granuloma, cytokine, Th1, Th2.

INTRODUCTION

Murine CD4$^+$ T helper (Th) cells are divided into Th1 and Th2 subclasses based on their patterns of cytokine secretion. Th1 cells secrete IL-2, IFN-γ and TNF-α; while Th2 cells secrete IL-4, IL-6 and IL-10. Differentiation into Th1 and Th2 subsets occurs through Th0 stage, where both Th1 and Th2 cytokines are released from the same cell[1].

In schistosome-infected animals, marked granulomatous reaction occurs around the eggs trapped in the intrahepatic and intestinal venules, and ultimately may lead to fibrosis. This is the main pathogenesis of schistosome infection[2]. In schistosomiasis mansoni, a great deal of research has been carried out on the mechanism and regulation of granuloma formation, which has been characterized as a CD4$^+$ T cell-dependent delayed-type hypersensitivity reaction, with Th2 responses playing a dominant role in the process[3,4]. However, less is known about schistosomiasis japonica, especially which Th subset is responsible for the granuloma formation induced by the eggs of *S. japonicum*.

Mice are permissive hosts for schistosomes and provide an important model for defining the immunopathogenesis of the disease and, furthermore, a system for assessing the impact of modulating the host immune response[5]. A model of synchronous pulmonary granuloma formation in response to the eggs of schistosomes was developed by von Lichtenberg[6] and has served as a useful *in vivo* model for studying the regulation of T helper subsets and their

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associated cytokines on granuloma formation[7,8]. It was previously reported that the size of granuloma induced by the eggs of *S. japonicum* differed according to the strains of mice[2]. In this paper, with the use of a pulmonary granuloma formation model, a comparative study was carried out on pulmonary granuloma formation and Th pattern among BALB/c, C57BL/6, C3H/He and ddY mice.

Materials and Methods

Animals

Female 8-wk-old BALB/c, C57BL/6, C3H/He and ddY mice were purchased from Japan SLC Co., Ltd. (Hamamatsu, Japan), with 5 mice per strain.

Eggs and antigen preparation

Eggs were extracted from the intestines and livers of ddY mice which were experimentally infected with cercariae of *S. japonicum* (Kofu strain) for two months as described[9]. Soluble egg antigen (SEA) was prepared as described in the preceding report[10].

Induction of pulmonary granuloma

The induction of synchronous egg-induced granulomas was performed as described by von Lichtenberg[6]. Each mouse was injected with 10,000 eggs of *S. japonicum* in PBS via the tail vein. Two weeks later, all mice were sacrificed under ether anaesthesia. Their left lungs were fixed with Bouin-Hollande fixative and processed for histological examination with H&E staining. The areas of granulomas containing a single egg were measured with Leica Quantimet 500 Image Analyzing System (Leica Cambridge Ltd., UK). For every mouse, three to five sections were read, twenty granulomas were measured and the mean area calculated.

Spleen cell preparation and cytokine assay

The spleen of each mouse was aseptically removed. Single cell suspensions were prepared by forcing minced spleen through a fine wire mesh, lysing erythrocytes with lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4), and washing with PBS. The spleen cells were suspended in RPMI-1640 medium supplemented with 10 % heat-inactivated FCS (Life Technologies, Inc., NY, U.S.A.), 2 mM glutamine, 1×10^{-5} M 2-ME, and 100 $\mu g/ml$ kanamycin. Cells (1.5×10^7 in 10 ml medium) were cultured in petri dishes without or with SEA (10 $\mu g/ml$) of *S. japonicum* at 37°C in 5 % CO_2 in a humidified atmosphere for three days. The supernatants were collected and stored at −80°C until use.

IL-2, IL-3, IL-4 and IL-6 were assayed by using CTLL-2, FDC-P2, CT4S, and 7TD1, cytokine-dependent cell lines respectively; IFN-γ and TNF-α were assayed by WEHI-279 and LP-3, cytokine-sensitive cell lines respectively. The growth of the cells except LP3 was measured by the MTT method[11]. LP3 cells (for TNF-α) were stained with crystal violet as described[12]. The cytokines were quantitated by reference to the standard curves derived from known concentrations of recombinant cytokines. If the result was positive, specific monoclonal antibody (mAb) was used to confirm the specificity. These were 11B11 for IL-4, 6B4 for IL-6, and MP2-8F8 for IL-3.

In vitro depletion of T cell subsets

To determine the T lymphocyte subsets responsible for the production of the Th2 type of cytokine (IL-4), the depletion of total T cell ($CD3^+$ cells), $CD4^+$ cells, or $CD8^+$ cells were performed. Briefly, spleen cells pooled from the egg-injected C3H/He mice were suspended in RPMI 1640 medium. Monoclonal antibodies to

mouse CD3, CD4, CD8a (Life Technologies) or normal rat IgG (as a control) were added to the cell suspension, with the addition of 10 % guinea pig serum (Denka Seiken Co., Japan) as a source of complement. The suspension was incubated at 37°C for 30 min, and then washed twice times with the medium. The treated spleen cells were cultured with SEA stimulation, and the supernatants were collected for cytokine assay as above.

Proliferation assay

Spleen cells (5×10^5 in 0.2 m*l*) were incubated for 72 hr in 96-well microtiterplates, stimulated with SEA (10 μg/m*l*), or ConA (2 μg/m*l*, Sigma Chemical Co., MO, U.S.A.), or without stimulation at 37°C in 5 % CO_2 in a humidified atmosphere. During the last 6 hr, 0.5 μCi of [^{3}H]-thymidine (DuPont NEC Products, MA, U.S.A.) was added to each well, and cells were harvested onto glass wool filters. [^{3}H]-thymidine incorporation into DNA was assayed by Liquid Scintillation Counter(Aloka Co., Ltd., Japan).

Statistics

Significance was analyzed by Student's t test and was determined as p value < 0.05.

Results

Size of granuloma

A representative granuloma induced by the injected egg in the lungs is shown in Fig. 1. Among BALB/c, C3H/He, C57BL/6 and ddY mice, the mean areas of granuloma were (mean $\pm$ SD, μm^2): 4926.6 $\pm$ 613.8, 4843.5 $\pm$ 473.3, 5478.2 $\pm$ 303.7 and 5026.9 $\pm$ 430.5 respectively. There was no significant difference among the four groups ($p > 0.05$).

Cytokine secretion

In the supernatants of cultured spleen cells,

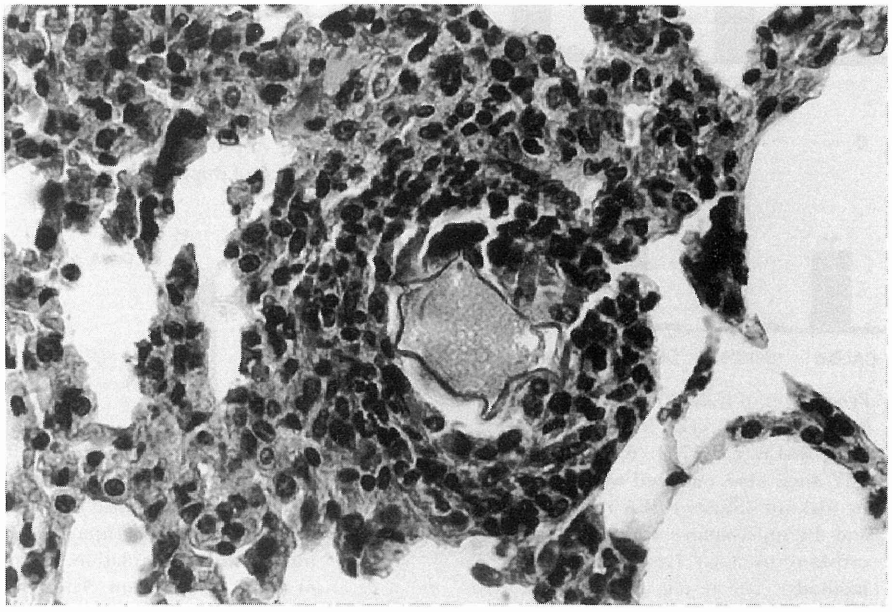

Fig. 1. A representative microscopic figure of pulmonary granuloma from C3H/He. A schistosome egg surrounded by granuloma composed chiefly of mononuclear cells and focally by granulocytes. H&E $\times$ 400.

IL-3, IL-4 and IL-6 were detected in all strains of mice. The spleen cells restimulated with SEA *in vitro* produced significantly higher levels of the above three cytokines than unstimulated cells ($p < 0.01$). C3H/He was the highest, while BALB/c was the lowest responder to the eggs (Fig. 2). IL-2, IFN-γ and TNF-α were undetectable in all strains of mice.

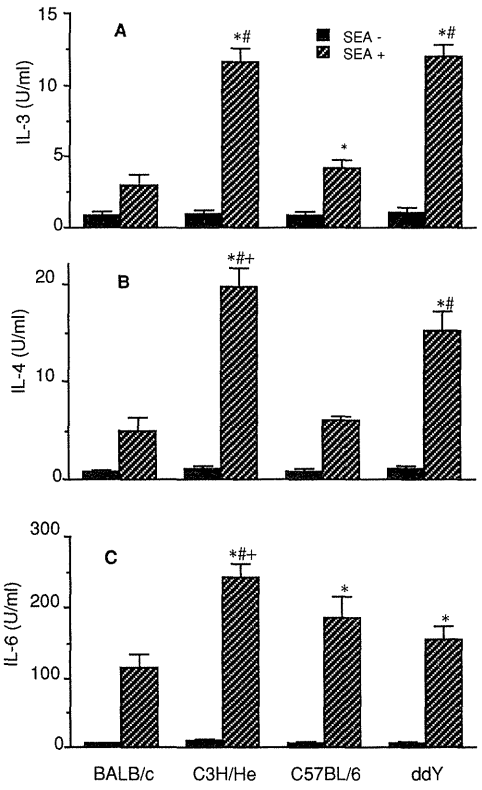

Fig. 2. Production of IL-3 (A), IL-4 (B) and IL-6 (C) by spleen cells. The spleen cells from BALB/c, C57BL/6, C3H/He or ddY mice were cultured with (SEA+) or without (SEA−) SEA respectively, and the supernatants were collected for cytokine bioassay (see materials and methods). The results are expressed as mean ± SD of 5 mice per group. Statistical significance: $p < 0.01$ compared with: BALB/c (*), C57BL/6 (#), ddY (+).

In vitro depletion of T cell subsets

As shown in Fig. 3, depletion of $CD3^+$ cells from the spleen cells of egg-injected C3H/He mice abrogated almost all the IL-4 response; depletion of $CD4^+$ cells abrogated most IL-4 production; while, the removal of $CD8^+$ cells had no such effect.

Proliferation response

The proliferation assay of spleen cells to SEA or ConA from egg-injected C3H/He mice was carried out (Fig. 4). The spleen cells proliferated modestly to SEA, with cpm about two folds higher than background. The spleen cells from naive mice also weakly responded to SEA.

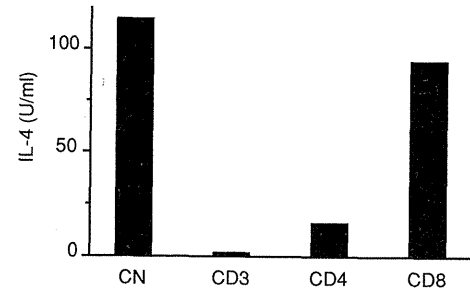

Fig. 3. IL-4 production by pooled spleen cells from C3H/He mice before depletion (CN) and after $CD3^+$, $CD4^+$, or $CD8^+$ cell depletion. The results are the means of duplicate assays.

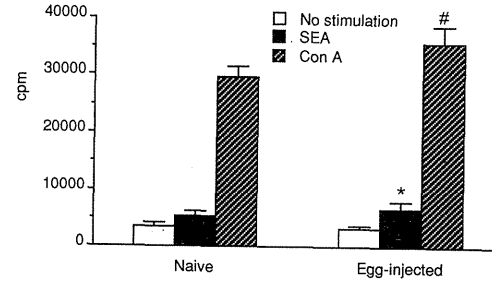

Fig. 4. Proliferation response of spleen cells from naive or egg-injected C3H/He mice without stimulation, or with SEA or ConA stimulation. The results are the means of triplicate assays from one representative experiment. Statistical significance: * $p < 0.05$; # $p < 0.01$ as compared with naive mice.

Discussion

In the present study, we compared granuloma formation and cytokine secretion following the injection of the eggs of *S. japonicum* among BALB/c, C3H/He, C57BL/6 and ddY mice. The results showed that spleen cells from C3H/He and ddY mice produced a higher level of IL-3 and IL-4 than BALB/c and C57BL/6. IL-6 was the highest in the supernatants of cultured spleen cells from C3H/He mice. The spleen cells produced much lower levels of IL-3, IL-4 and IL-6 when cultured without SEA restimulation, indicating that persistent stimulation with SEA *in vitro* was necessary for the production of the cytokines. IL-2, IFN-γ and TNF-α were undetectable in all the supernatants. By depletion of T cell subsets *in vitro*, it was demonstrated that $CD4^+$ T cells were responsible for the production of IL-4, one of the main Th2 cytokines. Besides cytokine secretion, spleen cells also proliferated modestly upon SEA stimulation. We previously reported that IL-5, one of Th2 cytokines could be detected at mRNA level by RT-PCR in the lungs of mice after injection of *S. japonicum* eggs, while Th1 cytokine IFN-γ and IL-12 were undetectable[13]. In our experimental infection model with *S. japonicum* in C3H/He mice (up to 20 weeks after infection), we found that at 3 weeks after infection, the cytokine secretion from spleen cells was similar to that of normal mice; at 5 weeks after infection, Th1 cytokines (IL-2, IFN-γ and TNF-α) were depressed and even became undetectable at 7 weeks after infection. Regarding Th2 cytokines, IL-4 reached to a peak in 7 weeks after infection, then gradually decreased but remained at much higher level than in normal mice (unpublished data). From these results, we can conclude that the Th response to the eggs of *S. japonicum* was Th2 dominant both in the pulmonary granuloma formation model and the experimental infection model. Our findings are consistent with the observation by Xu et al. in that Th2 cytokine production, characterized by IL-4 and IL-5, represented the major response after the eggs of *S. japonicum* laying began, while the Th1 function of IFN-γ and IL-2 production was greatly depressed[14]. In schistosomiasis mansoni, Cheever reported that granuloma formation, particularly in its growth and maintenance phase was largely associated with the increased level of Th2 cytokines after egg laying begins; while the Th1 response was down regulated[15].

In *Leishmania major* infection in mice, Th response differs according to the strains of mice. BALB/c is Th2 dominant and susceptible to the infection. While, C57BL/6 is Th1 dominant and is resistant to the infection[16,17]. From our results, Th response to the eggs of *S. japonicum* was Th2 dominant both in BALB/c and C57BL/6 mice, suggesting that murine Th response may differ according to the parasites infected. Although the development of granuloma is associated with vigorous Th2 response and suppressed Th1 response, the complex interrelation between Th1 and Th2 cytokine response to the induction and regulation of granuloma formation remains unclear.

In summary, it was suggested that the Th2 pattern of response could be induced to the eggs of *S. japonicum* in BALB/c, C3H/He, C57BL/6 and ddY mice.

The research reported herein was performed in accordance with the Guidelines for Animal Experiments, Yamanashi Medical University.

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