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ORIGINAL PAPER

Alteration of T cell subtypes in spleen and antibodies of serum in mice infected with *Angiostrongylus cantonensis*

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Abstract The immune responses of *Angiostrongylus cantonensis* infection are closely relevant to the host's self-protection and the nematode's pathogenesis. In the present study, BALB/c mice were randomly divided into uninfected control group, infection group 1, and infection group 2. The infection group 1 and infection group 2 were infected with 20 and 40 third-stage larvae of *A. cantonensis* per mouse, respectively. The splenocytes from the mice were collected and cultured on the 19th and 25th days post-infection; the subtypes of T cells in splenocytes were detected by flow cytometry with fluorescence staining method, and the cytokines in cultured supernatants of splenocytes were assayed by the method of ELISA. The specific IgG and IgE antibodies in sera of the mice were periodically detected by ELISA. The results showed that the percentages of $CD4^+$ and $CD4^+$ IL-4^+ T cells in splenocytes of infected mice were much higher ($P<0.05$) than those in control mice; however, the percentages of $CD4^+$ IL-17^+ and $CD4^+$ IFN-γ^+ T cell were much lower($P<0.01$) after the infection. The levels of $CD8^+$ T cells in infected mice also rose, but differences between control mice and infected mice were not significant. In comparison with control mice, the concentration of IL-4 in the cultured supernatants of splenocytes in infected mice increased significantly ($P<0.05$), but that of IL-17 decreased significantly ($P<0.01$). In addition, the number of larvae infected and days after infection may influence levels of the T cell subtypes and the cytokines in spleen, too ($P>0.05$). On humoral immunity, the levels of specific IgG antibodies in sera rose a bit at the fifth day post-infection, and reached a peak at the 20th day post-infection; the specific IgE antibodies gradually heightened during first 10 days post-infection; then, it showed a downward trend during the 15th to 25th days post-infection. It is evident that the percentages of $CD4^+$ T lymphocytes of spleen in the mice infected with *A. cantonensis* markedly increase and polarize to Th2 phenotypes, and the function of Th17 cells is inhibited. In addition, the elevation of specific IgG antibodies in sera of the infected mice is more significant than that of specific IgE antibodies.

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Introduction

Angiostrongylus cantonensis (*A. cantonensis*) is the pathogen of angiostrongyliasis, an emerging infection disease, which mainly invades human central nervous system and cause eosinophilic meningoencephalitis (Hüttemann et al. 2007). In recent years, several outbreaks of human angiostrongyliasis have been reported in China and other countries (Wang et al. 2008; Qu et al. 2007; Lindo et al. 2002; Slom et al. 2002; Ko et al. 1987), and its importance has been recognized. Previous studies (Sugaya et al. 1997; Perez et al. 1989) showed that the immune responses of the hosts infected with *A. cantonensis* were very important for the host's resistance to the infection and pathogenesis of the parasitic nematode (OuYang et al. 2012; Wei et al. 2012). Therefore, the alteration of T cell subtypes in spleen and antibodies of serum in mice infected by *A. cantonensis* were observed in this study.

Table 1 Flow cytometric analysis of spleen T cells

	Control (%)	Infection 1 (%)	Infection 2 (%)	*P*
$CD4^+$ T	15.890±6.019	21.318±6.905	25.252±10.161	<0.05
$CD8^+$ T	2.511±0.604	3.250±1.311	3.759±1.579	>0.05
$CD4^+$ IL-4^+ T	0.704±0.253	2.134±0.801	2.496±1.529	<0.05
$CD4^+$ IL-17^+ T	1.181±0.261	0.633±0.163	0.651±0.297	<0.01
$CD4^+$ IFN-γ^+ T	12.466±2.687	5.798±2.389	5.658±3.080	<0.05

Materials and methods

Mice

Female BALB/c mice aged 6 weeks, SPF grade, were purchased from Experimental Animal Center, Guangdong Province.

Reagents

PMA, Saponing, Ionomycin, and Brefeldin A (BFA) were produced by Sigma Company, USA. PerCP anti-mouse CD4, FITC anti-mouse CD8, APC anti-mouse IL-4, APC anti-mouse IFN-gamma, and PE anti-mouse IL-17 were purchased from BD Biosciences Inc. USA. IL-4 (DY404) and IL-17 (DY421) ELISA assay kit were purchased from R&D Systems Inc., USA.

Instruments

Flow cytometry (Calibur) from BD Biosciences Inc., EL×800 microplate reader from BioTeK Inc., 1267P CO_2 incubator manufactured by Yi Liang medical company, Shanghai, China.

Methods

Animal infection

The third-stage larvae of *A. cantonensis* were obtained from snail *Achatina fulica* (Chen et al. 2011). Thirty BALB/c mice were randomly divided into control group, infection group 1, and infection group 2; each group has ten mice. The infection group 1 was infected with 20 larvae per mouse by peritoneal injection, infection group 2 infected with 40 larvae, and control group received only water.

Confirmation of infection

The activities of infected mice were observed during the experiment. The larvae of *A. cantonensis* in the brain of infected mice were examined by microscopic or visual study. The pathological changes of the brain tissues were detected by paraffin section and HE staining.

Collection of sera

The blood was periodically collected from the caudal vein or eyeball of the infected mice at pre-infection and post-infection, and the sera were stored at −20 °C.

Collection of splenic lymphocytes

Five mice in each group were sacrificed at the 19th and 25th days post-infection. Splenic lymphocytes of the mice were collected as described in the report (Anukumar and Shahir 2011). The cells were resuspended in 4 mL RPMI Medium

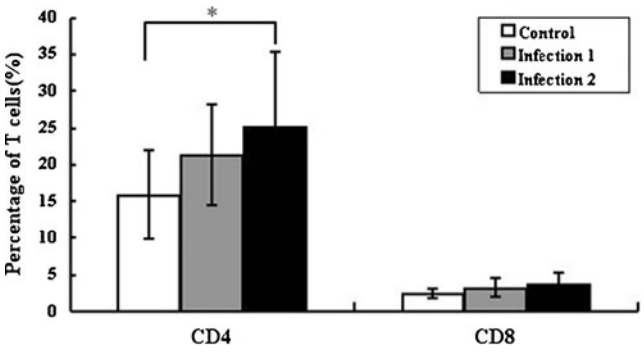

Fig. 1 Percentage of $CD4^+$ T and $CD8^+$ T cells in spleen. (*P<0.05)

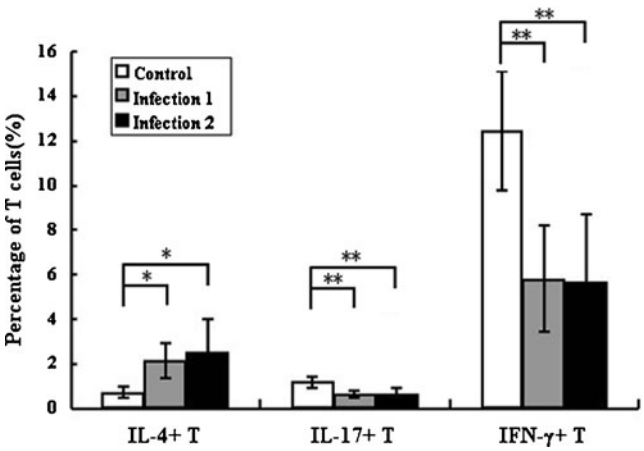

Fig. 2 Percentage of IL-4^+ T, IL-17^+ T and IFN-γ^+ T cells in spleen. (*P<0.05; **P<0.01)

Table 2 The determinations of the cytokines in culture supernatants of splenocytes

	Control (pg/mL)	Infection 1 (pg/mL)	Infection 2 (pg/mL)	*P*
IL-4	215.203±158.131	453.427±246.077	469.421±221.374	<0.05
IL-17	378.474±152.568	97.846±47.703	67.360±31.591	<0.01

1640 (1×) liquid, stained by 0.4 % trypan blue and counted under microscope.

Detection of T cell subtypes in splenocytes

According to the methods reported by other authors (Anukumar and Shahir 2011; Yang et al. 2006), spleen lymphocytes(2×10^6 per milliliters) were incubated with PMA 10 ng/mL and Ionomycin 1 μg/mL in 37 °C, 5 % CO_2 incubator for 1 h and then incubated with BFA for 4 h. The cells were fixed by 4 % formaldehyde and added buffer (including Saponin) at 4 °C overnight. The mAbs labeled fluorescein were mixed with the cells; the subtypes of T cells in splenocytes were detected by flow cytometry.

Detection of cytokines in culture supernatants of splenocytes

The splenocytes were incubated with PMA and Ionomycin in the cell culture plates for 72 h in 37 °C, 5 % CO_2 incubator; each splenocyte suspension was simultaneously cultured with three well. The concentrations of IL-4 and IL-17 in culture supernatants of splenocytes were assayed by ELISA according to the protocol in kit. The optical density (OD) values were taken the average detected value of the three well; then, OD values were converted into concentration of the cytokine in accordance with the standard curve.

Preparation of the adult antigens

The preparation of adult *A. cantonensis* antigens for ELISA is referred to the report of Jin (Jin et al. 2006).

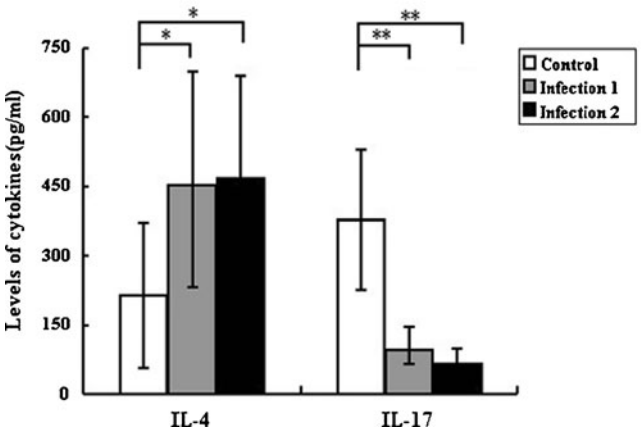

Fig. 3 Cytokines IL-4 and IL-17 in culture supernatants of splenocytes

Assay of specific IgG antibodies in sera

Microtiter plates were coated with the adult *A. cantonensis* antigens (10 μg/mL, 100 μL per well). The dilution of the mouse sera tested was 1:25, and that of the anti-mouse IgG-HRP antibody (Jackson ImmunoResearch Inc., USA) was 1:5,000 in ELISA assay. The optical density (OD) value was read at 450 nm with a microplate reader.

Assay of specific IgE antibodies in sera

The dilution of anti-mouse IgE-HRP antibody (Alpha Diagnoestic International Inc., USA) was 1:4,000, and the other methods were similar to the detection of specific IgG antibodies.

Statistical analysis

All data were expressed as mean±SD. The comparisons between infection groups and control group were made with SPSS, version 19.0 (SPSS Inc., Chicago, IL, USA), and statistical methods used in data analysis include analysis of variance, *t* test, and Wilcoxon rank sum test.

Results

Flow cytometry of spleen T cells

The percentages of spleen T cells from the control group and infection groups in flow cytometry were shown in Table 1.

$CD4^+$ and $CD8^+$ T cells

The changes of $CD4^+$ or $CD8^+$ T cell ratios after infection were shown in Fig. 1.

T cell subtypes in splenocytes

The changes of T cell subtype ratios after infection were shown in Fig. 2.

Cytokines in culture supernatants of splenocytes

The detective results of cytokines IL-4 and IL-17 in culture supernatants of splenocytes from the control mice and infected mice were shown in Table 2 and Fig. 3.

Table 3 The examinatorial results of the infected mice in different times after infection

Flow cytometry (%)					ELISA(pg/mL)	
Time	$CD4^+$ T	IL-4^+	IL-17^+	IFN-γ^+	IL-4	IL-17
19th day	24.455±8.165	1.808±0.821	0.578±0.252	5.607±3.697	462.623±148.482	84.411±30.902
25th day	22.115±8.670	2.725±1.781	0.701±0.205	5.844±1.779	460.227±309.511	80.805±37.213

Alteration of T cells and cytokines in different times after infection

Flow cytometry analysis

As shown in Table 3, the percentages of $CD4^+$ T, $CD4^+$ IL-4^+ T, $CD4^+$ IL-17^+ T, and $CD4^+$ IFN-γ^+ T cells in the 19th and 25th days post-infection ($P>0.05$).

Detection by ELISA

As shown in Table 3, the levels of IL-4 and IL-17 in cultured supernatants of splenocytes in the 19th and 25th days post-infection ($P>0.05$).

Dynamics of specific antibodies in serum

IgG antibody

The results were shown in Fig. 4. The levels of specific IgG antibodies in sera of infected mice rose a bit at the fifth day post-infection, the levels of the antibodies reached a peak at the 20th day post-infection.

IgE antibody

The results were shown in Fig. 5. The level of specific IgE antibodies in sera of infected mice arrived at a peak during the 10th to 15th days post-infection; then, it showed a downward trend during 15th to 25th days post-infection.

Confirmation of infection

Some symptoms being relative to central nervous system were damaged, e.g., spin, paralysis had begun to appear in the infected mice since 16th day post-infection. The lesions of hyperemia, hemorrhage, and tissue adhesion on surface of brain tissue could be seen in the infected mice. The pathological changes of the brain tissue were shown in Fig. 6. The larvae of *A. cantonensis* could be obtained in the brain of all infected mice.

Discussion

T cells (T lymphocytes) are divided into $CD4^+$ and $CD8^+$ T cells. The biological function of $CD4^+$ T cells is to stimulate and modulate the host immune response, and $CD8^+$ T cells can specifically kill target cells. In this study, the results of flow cytometry showed that percentages of $CD4^+$ T cell in splenocytes of infected mice were significantly higher than those in control mice. In addition, the levels of $CD8^+$ T cells in infected mice increased, but differences between control mice and infected mice were not significant. It indicates that $CD4^+$ T cells may play a major role in immune response of the early-phase infection. The results are consistent with other reports (Lee et al. 1996; Aoki et al. 1998).

$CD4^+$ T cells (or helper T cells, Th) mainly consist of Th1 and Th2 cells, both usually secrete different cytokines to have mutual antagonism in immune response. Therefore, the cytokines from dominant Th cell type will determine the development and outcome of infection (Mosmann et al.

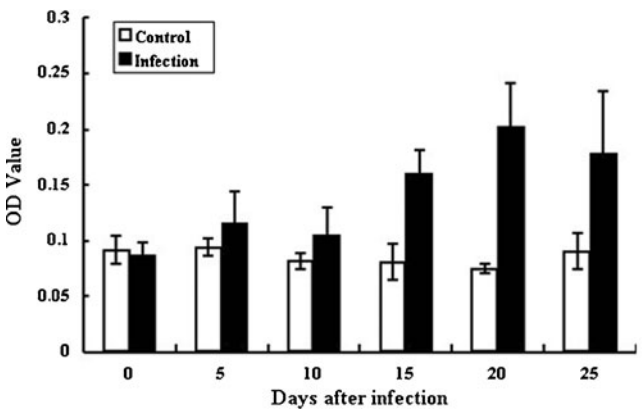

Fig. 4 Detection of the specific IgG antibodies in sera

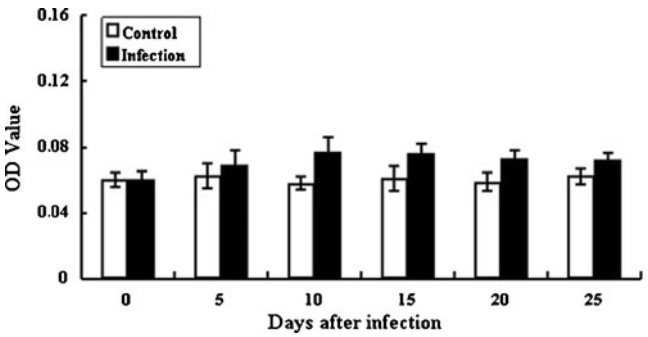

Fig. 5 Detection of the specific IgE antibodies in sera

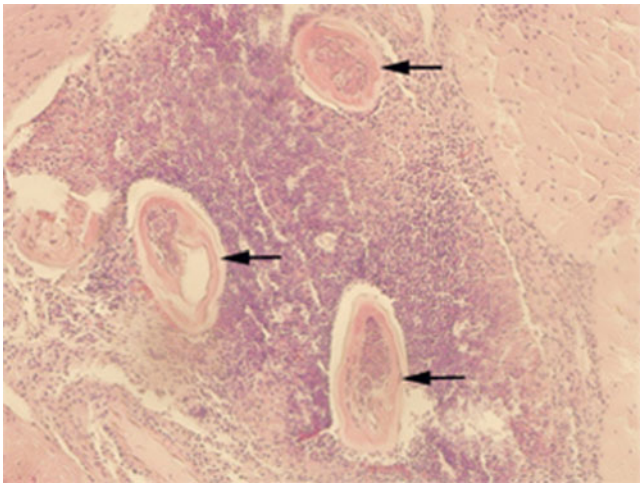

Fig. 6 Pathological section of brain tissue from the infected mice (*arrows* indicate cross section of *A. cantonensis*)

1986; Abbas et al. 1996; Chen 1998; Chen and Wang 2009). IL-4 is produced by $CD4^+$ $IL\text{-}4^+$ T cells (belong to Th2); its main biological functions are to proliferate the B cell, to induce antibody types transition to IgE, and to promote the proliferation and function of Th2 cells by autocrine effect. The results of flow cytometry showed that percentages of $CD4^+$ $IL\text{-}4^+$ T cells of infected mice were significantly higher than those of the control mice, and levels of IL-4 in cultured supernatants of splenocytes were also significantly elevated after the infection. It is approximately consistent with results of the previous research (Sugaya et al. 1997).

Cytokine IL-17 is mainly generated by Th17 cell belonging to $CD4^+$ T cell (Chen 1998). IL-17, involved in inflammation and vascular generation, was paid more attention in recent years (Kolls and Linden 2004), but its changes in *A. cantonensis* infection were not known yet. In this study, the percentage of spleen $CD4^+$ $IL\text{-}17^+$ T cells and concentration of IL-17 in cultured supernatants of splenocytes from the infected mice were significantly lower than those of the control mice. Another research (Harrington et al. 2005) showed that IL-4, which was high expression after schistosome infection, could inhibit the differentiation of Th17 cells and IL-17 secretion by the PKC-θ signal pathways. Whether IL-4, which was also high expression after *A. cantonensis* infection, restricts the mature of Th17 and production of IL-17 of the infected mice requires further investigation.

IFN-γ, produced by $CD4^+$ $IFN\text{-}\gamma^+$ T cell being Th1, chiefly takes effects on killing intracellular parasitic protozoan in the infection of parasites (Kushawaha et al. 2011; Langermans et al. 1992; Su and Stevenson 2000). The results of this study showed that the ratio of $CD4^+$ $IFN\text{-}\gamma^+$ T cells in the infected mice was significantly lower than that of the control mice. The reason may be due to significant increasing of $CD4^+$ $IL\text{-}4^+$ T cells and IL-4 levels in the mice infected with *A. cantonensis*, because Lee's study (Lee et al. 1996) showed that IL-4 could suppress the differentiation and proliferation of Th1 cells by inhibiting the expression of STAT1.

The experimental results showed that levels of the T cell subtypes and cytokines in spleen between infection group 1 and infection group 2 were different, and there were also differences between 19th and 25th days post-infection, but the differences were not statistically significant. It suggests that number of larvae given and infective time might finitely affect the immune responses of the infected mice.

IgG, which is the primary antibodies in host's immune response, plays a key role in the process of anti-infection immunity. At present, the information on dynamics of specific IgG antibodies in serum after *A. cantonensis* infection are mostly from the studies of infected rat as animal model (Jin et al. 2006; Huang et al. 2001; Pan et al. 2000). Rats and mice are discrepant in compatibility to *A. cantonensis*, but human being and mice seem to be same in this aspect. Therefore, the immunological observation to the infected mice as animal model may be more valuable in medicine. However, in our research, the dynamics of specific IgG antibodies in the infected mice was basically similar to the results in rats.

IgE (its Fc fragment) binding the FcεR I receptor in surface of eosinophil will lead to eosinophil degranulation and cause a series of biological effects. Some clinical studies (Dorta-Contreras et al. 2005; Padilla-Docal et al. 2008) found that the cerebrospinal fluids and sera of meningoencephalitis patients infected by *A. cantonensis* contained a high-level IgE. Meanwhile, the researches about *Schistosoma mansoni* (Gounni et al. 1994; Joseph et al. 1983; Dunne et al. 1992; Pinot de Moira et al. 2010) and *Trichinella spiralis* (Watanabe et al. 2005; Gurish et al. 2004) showed that high levels of IgE in the hosts might promote the elimination of the worms and reduce the chances of host's re-infection. In the present study, the levels of specific IgE antibody in sera of the mice at post-infection were higher than at pre-infection. If the phenomenon is associated with high levels of IL-4 in the infected mice and if the specific IgE can help the hosts in the immunity against *A. cantonensis*, it is interesting.

In summary, the ratio of spleen $CD4^+$ T cells of mice infected with *A. cantonensis* increases significantly, Th2 polarization appears in the immune responses, and the function of Th17 cells is inhibited. On humoral immunity, the levels of specific IgG and IgE antibodies in sera of the infected mice all increase significantly.

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