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Flow Cytometric Characterization of Lymphocyte Subpopulations in Mice Infected with *Clonorchis sinensis*

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Abstract: A Recent discovery of surface antigens in cells has led to the success of quantitative measurement of T-cell subpopulations, and this has especially opened the way for an epoch-making development in the understanding and classification of cellular immune mechanisms. It is known that phenotypes of T-cell subpopulations exist in many forms according to the variation of species or animal experimental models. In Korea, *Clonorchis sinensis* still gives rise to public concern as it infects more than eighty million people and threatens the public by causing cirrhosis of the liver, or liver cancer when liver infection becomes prolonged and chronic. Up until now there has been much progress in research and improvement in the classification system of *Clonorchis sinensis* in the area of humoral immunity, but as for research in the area of cellular immune mechanisms, there is almost none. Knowing all these circumstances, the authors delved for the characterization of lymphocyte subpopulations with mice as Clonorchis sinensis in the area of cellular immunity, and obtained the following results. That is, we injected Clonorchis sinensis antigens mixed in Freund's ajuvant solution intraperitoneally in mice and measured the T-cell subpopulation characterization of spleen lymphocytes with flow cytometry. The results of these measurements showed that CD2, CD5 and CD8 decreased early following injections but then increased again seven weeks after the injections. CD4, however, showed a slight increase shortly after the injection but then a fair increase seven weeks after the injection.

Key Words: *Clonorchis sinenesis*, T cell subpopulation, monoclonal antibodies, CD2, CD4, CD5, CD8, flow cytometry, mouse.

INTRODUCTION

When they are infected by parasites, both cellular immunity and humoral immunity take place and either kill parasites or suppress the comfort of parasitism[17]. The helminthes go through several stages of growth within the body of the hosts and reach their parasitic places, and it is known as that, due to the fact that the characteristics of the antigens undergo changes is keeping with the growth stages of the parasites, at this moment antigens secreated from the bodies of the helminthes activate the actions of lymphocytes, mast cells and granulocyte, and therefore regulate the inflammatory reactions of the tissues[13]. Yong et al.(1987) reported that humoral immunity reacted to the infections, by observing that antibodies IgE and IgG within serum increased when animal models were infected by *Clo-*

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norchis sinensis and *Paragonimus westermani*. Colley(1971) and Ahn and Colley(1984) observed that when animal models infected to Schistosoma mansoni were stimulated by the antigens of the parasites, the blastogenesis reaction of lymphocytes and delayed hypersensitivity were induced. Oldham and Williams(1985) reported that cellular immunity reacted to the parasite infections, by observing the increase of interleukin-2(IL-2) when the animal models were infected by *Clonorchis sinensis*. Yong et al.(1987) had once reported that antibody dependent cellular immunity had something to do with killing parasites displayed a cellualr toxicity of mast cells toward the metacercariae of *Paragonimus westermani* in vitro. Presently many researches on classify the cellular mediated immune reaction to each parasite are being conducted and many notible facts are being reported. Especially researches or immune functions of T cells and also on finding out the presense of phenotypes of T cells for each monoclonal antigen are necessary for classifying immune mechanism of the cells in coming years. But for the case of Clonorchis sinensis, the mechanism for the cellular immune reactions has not been sufficiently cleared out yet, especially due to the fact that the knowledge on the immune characteristics of each experimental animal model and the fundamental data supporting it are few, a difficulty arises in obtaining an objective explanation for the experimental results, also a confusion might be caused due to the mistake of the non-objective explanation of the experimental results.

This experiment was conducted to find out the immune characteristics of T cell in mice by observing the immune characteristics of the cells against lymphocyte subpopulation cells inside the mouse spleen and especially subpopulation cell reactions to the antibodies of monoclonals when somatic antigens of *Clonorchis sinensis* together with Freund's adjuvants(FA) were injected into the mouse abdominal cavity. Therefore, the purpose of this experiment lies for the future usage of these experimental results as some fundamental data in this field.

MATERIALS AND METHODS

Preparation of the worm antigens

The metacercariae of *C. sinensis* were obtained from naturally infected *Pseudorasbora parva* collected in Kimhae-Gun, Korea. Rabbits were infected with oral feeding of 1,000 metacercariae and were sacrificed on the third month of experimental infection. The collected worms were washed three times with physciological saline. The suspensions of *C. sinensis* in sodium phosphate buffer(0.01 M, pH 7.4) was sonicated 4 times with 5-10 pulses at 100 watts for 15 seconds(Blackstone Sheffield,U.S.A). The homogenates was then centrifuged at 10,000 g for 15 minutes. The supernatant was used as a crude antigens throughout this experiment. Protein concentration was determined by method of Lowry et al.,1951.

Experimental animal of immunization

Six-week old BALB/c mice were immunized with water-soluble crude C. sinensis adult worm antigens. They were injected intraperitoneally(i.p.) with 100μg of antigens mixed in Freund's adjuvant complete(FAC) were i.p. injected. Two weeks after the second immunization, third immunization was conducted with 100 ug of antigen intravenously.

Analysis of splenic T lymphocyte subpopulations by flow cytometry

Three days after third immunization, the spleen of a mouse was removed aseptically, and spleen cells were obtained. Spleen was chopped in PBS containing 0.1% sodium azide. Cell suspensions were layered over Ficoll-paque and centrifuged at 400$\times$g for 30 min. Mononuclear cells were collected and

washed 3 times in PBS. Spleen cells were used at a concentration of 2×10^6 /ml. For detection of T cell subsets, the following monoclonal antibodies were used; Fluorescein isothiocyanate(FITC) conjugated rat anti-mouse CD2(LFA-2), fluorescein isothiocyanate(FITC) conjugated rat anti-mouse CD4(L3T4) monoclonal antibody, fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD5(Ly-1) monoclonal antibody, fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD8a(Ly-2) monoclonal antibody were used. All reagents were purchased from Pharmingen, USA. To 20 l cell suspension(2×10^6/ml), each of above mentioned monoclonal antibodies were added to detect individual cellualr subpopualtion.

The cells were incubated for 30 min at 4'C and washed 2 times in PBS and then fixed with 1% paraformaldehyde. The number of cell populations were determined by flow cytometric analysis using FACStar(Becton Dicton, USA).

RESULTS

CD2(LFA-2)

For the CD2 subcells from the mice in the control group its experimental result of 97±2% was obtained as for the CD2 from the mice which after having been injected with the mixture of *Clonorchis sinensis* antigens and FCA were kept for one week its experimental result decreased rapidly to 11±3%. But for the CD2 from the mice which after having been injected with the mixture of *Clonorchis sinensis* antigens and Freund's adjuvant incomplate(FAI) were kept for one week its experimental result increased to 90±4%. and lastly for the CD2 subcells from the mice which were injected with *Clonorchis sinenesis* antigens only its experimental result increased to 99±4%(Table 1).

CD4(L3T4)

For the CD4 subcells from the mice in the control group its experimental result of 32±2% was obtained as for the CD4 subcells from the mice which after having been injected with the mixture of *Clonorchis sinensis* antigens and FAC were kept for one week its experimental result showed a high increase to 60±4%. For the CD4 subcells from the mice which after having been injected with the mixture of CSA and FAI were kept for one week its experimental result showed a decrease to 15±1%. And lastly for the CD4 subcells from the mice which had been injected with CSA only its experimental result showed 21±4% (Table 1).

CD5(Ly-1)

For the CD5 subcells from the mice in the control group its experimental result showed 74±2% as for the CD5 subcells from the mice which after having been injected with the mixture of CSA and FAC were kept for one week its experimental result showed a very low percentage of 8±4%. For the CD5 sub-

Table 1. Percentage of T cell subpopulations in the spleen of mice infected with *Clonorchis sinenesis* by flow cytometry

mAb	Cont.	Days after *Clonorchis sinensis* infection		
		1st immunization	2nd immunization	3rd immunization
CD2 (SFA-2)	97±2	11±3	90±4	99±4
CD4 (L3T4)	32±2	60±4	15±1	21±4
CD5 (Ly-1)	8±4	8±4	47±1	97±4
CD8 (Ly-2)	14±2	1±2	7±2	28±4

1st immunization: antigen(100ug) in Freund's complete adjuvant. 2nd immunization: antigen(100ug) in Freund's incomplete adjuvant. 3rd immunization: antigen(100ug) only. cont.: control group. mAb: mouse monoclonal antibodies.

cells from the mice which after having been injected with the mixture of CSA and FAI were kept for one week its experimental result increased to 47±1%. And lastly for the CD5 subcells from the mice which were injected with CSA only its experimental result showed a increase of 97±4%(Table 1).

CD8(Ly-2)

For the CD8 subcells from the mice in the control group its experimental result showed 14±2% as for the CD8 subcells from the mice which after having been injected with the mixture of CSA and FAC were kept for one week its experimental result showed a low percentage of 14±2%. For the CD8 subcells from the mice which after having been injected with the mixture of CSA and FAI were kept for one week its experimental result increased to 7±2%. and lastly for the CD8 subcells from the mice which were injected with CSA only its experimental result increased to 28±4%(Table 1).

DISCUSSION

Its was reported that, when the mice had been infected with the parasites such as *Fasciola hepatica, Schistosoma mansoni, Taenia taeniaeformis, Trypanosoma,* and so on, experimentally, the increase of T lymphocytes was supressed in the early time during the infection period[9.12.16.26]. Wongreatanacheewin et al.(1987) observed that the suppression on the increase of T lymphocytes recovered after having infected hamsters with *Opisthorchis viverrini* and conducted a drug treatment, and therefore reported that the immunosuppression recovered again in the body of the hosts when the parasites were killed. As being seen here, it was known that the suppressive T lymphocytes and also mast cells play the role to suppress the increase of T lymphocytes[1.9.16]. In the early time of the infection period when the mice were infected with Toxoplasma gondii it was known that the cellular immunity played a very important role on the defense mechanism and it was reported that both the weight and the number of cells of the spleen increased 3-4 times, the number of monocytes increased 30 times, while the number of whole T cells and helper/inducer T cells decreased[7,8,14], within sera of the hosts infected by parasites. It was reported that the immune complex which was comprised of the antigens and antibodies of the parasites acted on the FC receptor on the surface membrane of the hosts' lymphocytes and therefore suppressed the reaction to mitosis of T lymphocytes*2,20)*. Cottrell et al., 1980a & 1980b) reported that as being stated above, the immunosuppressive elements which are present in sera are a high polymer fraction and have characteristics of being heat stable and non dialyable. That is non specific or specific immune complexes exist in the infected blood serum and supress the lymphocyte blastogenesis reactions[11]. Of all different experimental conditions, when both CSA and FA were injected, the number of lymphocytes of the mouse spleen decreased rapidly in the early time of the injection(3 weeks later) and after that their number increased gradually and 7 weeks after the injection; the highest increased number of lymphocytes was shown (Table1, Fig.1). In addition, Carvalbo et al.,(1985) reported the difference in the degrees of the parasitic diseases in the case for the *Lishimania* patients in their many different parasitic stages, That is, different degrees of the increase of the lymphocytes for many different stages of Lishimania antigens were observed. And also, Yamashita and Boros(1990) reported that in the case for *Schistosoma mansoni*, eggs antigens played a role in the increase of CD4 in the spleen. In this experiment, as the result from an injection of somatic antigens of *Clonorchis sinensis*, the number of CD4 increased after 3 weeks, decreased rapidly after 5 weeks, and showed an almost equal experimental

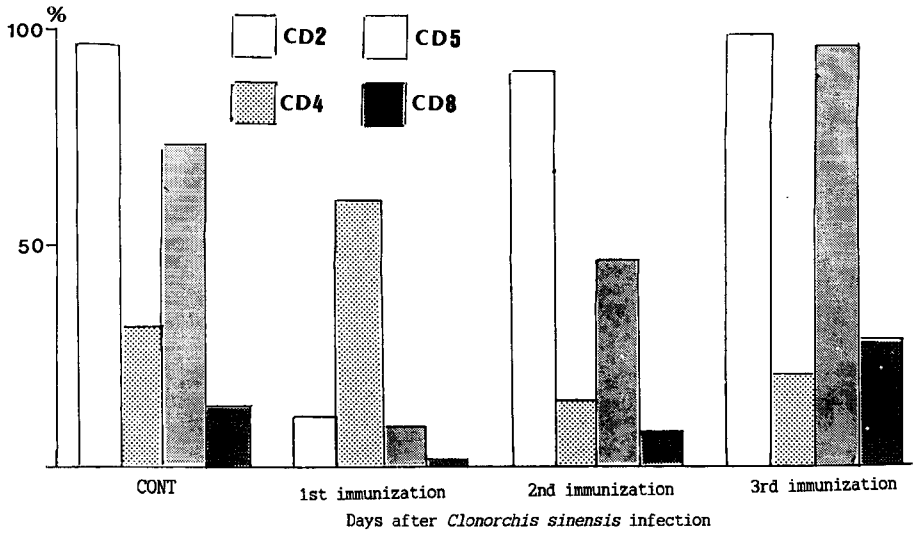

Fig. 1. Flow cytometric analysis of splenic lymphocytes with the monoclonal antibodies following *Clonorchis sinensis* infection.

result as the other groups after 7 weeks. As this result tells of the somatic antigens, further experiments about the increase or decrease of the number of antigens with eggs antigens of *Clonorchis sinensis* injected are needed in the future. As cytotoxic/suppressor T cells can measure both cytotoxic cells and suppressor cells which have reported that an immune unbalance which occured after infection with *Toxoplasma gondii* was heavily related to the increase of the number of suppressor T cells and their activities[8,10,24], as well the increase of the number of suppressor T cells, monocytes, and natural killer cells was heavily related to the clinical symptoms of the disease[18]. And Gazzinelli et al.(1991) reported that in the case for the chronic *Toxoplasma gondii* disease CD8± (cytotoxic) T cells and CD4±(helper/induce) T cells played an important role on the immune defense mechanism. Also in this experiment a result was observed that CD4, CD5, CD8, as well as CD2 had an influence to the increase or decrease of lymphocyte subpopulation of the mouse spleen for *Clonorchis sinensis*(Table 1, Fig. 1). In this experiment of both CSA and FA being injected, for the case of CD4 its result displayed an increase in the eary time of the injection while for the case of different monoclonal antigens their results showed an abrupt decrease in the early time of the injection and re-increase after 7 weeks. Upon the experimental results discussed above, when the mice were injected with both *Clonorchis sinensis* somatic antigens and FA into their intraperitoneally on various time spans, it was observed that the number of the spleen lymphocyte subpopulations decreased in the early time of the infection while after 7 weeks the number increased to the level of the other experimental groups or more.

REFERENCES

1. Baird LG and Kaplan AM(1977): Macrophage regulation of mitigen-induced blastogenesis. I. Demonstration of inhibitory cells in the spleens and peritoneal exudates of mice. *Cell Immunol*, **28:** 22-35.
2. Bout D, Dessant JP, Dupas H, Yazzabal L and Capron A(1977): Characterization of allergen in Schistosoma mansoni, Fasciola hepatica and *Echinococcus granulosus.*

Ann Immunol, **128(c):** 687-698.

3. Carvalho EM, Johnson WD, Barreto E, Marsden PD, Costa JLM Reed S and Rocha H(1985): Cell mediated immunity in american cutaneous and mucsal *lishimaniasis. J Immunol*, **135:** 4144-4148.
4. Colley DG(1971): Schistosoma egg antigen-induced lymphocyte blastogenesis in experimental murine *Schistosoma mansoni infection. J Immunol*, **107:** 1477-1480.
5. Cottrell BJ, Humber D and Sturrock RF (1980a): An immune suppressive factor in serum of patients with *schistosomiasis.* Trans Roy Soc Trop Med Hyg, **74:** 415-416.
6. Cottrell BJ, Sturrock RF and Vanhoegaerden M(1980b): An immunosuppressive factor in the serum of baboons(*papio anubis*)infected with *shistosoma mansoni. Immunology*, **39**: 589-598.
7. Gazzinelli RT, Xu Y, Heieny S, Cheever A and Sher A(1992): Simultaneous depletion of CD 4+ and CD8+ T lymphocytes is requred to reactivate chronic infection with *Toxoplsma gondii. J Immunol*, **149:** 175-180.
8. Goyal M, Ganguly NK and Mahajan RC (1989): Immunological response in experimentally reactivated *toxoplasmosis* in mice. *Med Microbiol Immunol*, **178:** 269-278.
9. Jayawardena AN and Waksman BN(1977): Suppressor cells in experimental *Trypanosomiasis. Nature*, **265:** 539-541.
10. Jones TC, Alkan S and Erb P(1986): Spleen and lymph node cell population in vitro cell proliferation and interferon production during the promary immune response to *Toxoplasma gondii* parasite. *Immunology*, **8:** 619-629.
11. Kamal KA and Higashi GI(1982): Suppression of mitigen induced lymphocyte transformation by plasma from patients with hepatosplenic *Schistosoma mansoni*: role of immune complexes. *Parasite Immunol*, **4:** 283-298.
12. Ketonja T, Hammerberg C and Schuring G(1987): Evaluation of spleen lymphocyte responsiveness to a T-cell mitogen during early infection with larval *Taenia taeniaeformis. Parasitol Res*, **73:** 265-270.
13. Lightowlers MW and Rickard MD(1988): Excretory-secretory products of helminth parasite: effects on host immune responses. *Parasitology*, **96:** S123-S166.
14. McLeod R, Eisenhauer P, Mack D, Brown C, Filice G and Spitalng G(1989): Immune reponses associated with eary survival after peroral infection with *Toxoplasma gondii. J Immunol*, **142:** 3247-3255.
15. Oldham G and Williams L(1985): Cell mediated immunity to liver fluke antigens during experimental *Fasciola hepatica* infection of cattle. *Parasite Immunol*, **7**: 503-516.
16. Pelley RD, Ruffier JJ and Warren KS (1976): Suppressive effect of a chronic helminth infection, *Schsitosoma mansoni,* on the in vitro responses of spleen and lymph node cells to the T cell mitogens phytohemagglutinin and concanavalin. *Inf Immuno,* **13:** 1176-1183.
17. Roitt IM, Brostoff J and Male DK(1989): *Immunology(2nd ed.)*. pp.9.8-9.10 & 17.4-17.6., Gower Medical Publishing. London. New York.
18. Sklenar I, Jones TC, Alkan S and Erb P (1986): Association of symptomatic human infection with *Toxoplasma gondii* with imbalance of monocytes and antigen-specific T cell subsets. *J Infect Dis*, **153:** 315-324.
20. Stout DR and Herzenberg LA(1975): The Fc receptor on thymus derived lympho-cytes II. Mitigen responsiveness of T lymphocytes bearing the Fc receptor. *J Exp Med*, **142:** 1041-1051.
22. Wongratanacheewin S, Rattanasiriwilai W, Priwan R and Sirisinha S(1987): Immuno-depression in hamsters experimentally

infected with *Opisthorchis viverrini. J Helminthol*, **61:** 151-156.

23, Yamashita T and Boros DL(1990): Changing patterns of lymphocyte proliferation, IL-2 production and utilization and IL-2 receptor expression in mice infected with *Schistosoma mansoni. J Immunol*, **145**: 724-731.

24. Yano A, Norose K and Yamashita K (1987): Immune response to Toxoplasma gondii analysis of suppressor T cells in a patient with symptomatic acute *toxoplasmosis. J Parasitol*, **73:** 954-961.

25. Yong TS, Kim TS, Lee JS, Lee OY and Kim DC(1987): Detection of circulating antigens in rats experimentally infected with *Paragonimus westermani* by ELISA. Korean *J Parasit*, **25:** 41-148.

26. Zimmerman GL, Kerkvliet NI, Brauner JA and Cerro JE(1983): Modulation of host immune responses by *Fasciola hepatica*: Responses of peripheral lymphocytes to mitogens during liver fluke infections of sheep. *J Parasit*, **69:** 473-477.

=국문초록=

간흡충 항원에 의한 마우스 비장 림프구의 아형 특성

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최근 세포 표면의 다양한 항원들이 발견되고 T세포 아형에 대한 정량적 측정이 가능하게 됨에 따라 세포성 면역기전의 규명과 이해에 획기적인 진전을 보이고 있다. T세포 아형의 phenotype은 실험동물의 모델(model)이나 종(species)에 따라서 다양하며 차이가 나는 것으로 알려져 있다. 우리나라의 경우 아직도 80만명 이상의 국민이 간흡충에 감염되어 있을 것으로 추정되는 문제의 기생충이며 감염상태가 만성적일 때에는 간경화나 간암으로 까지 진행되는 흡충으로서 이에 대한 대책이 요구되고 있다. 그동안 간흡충의 면역에 대한 연구는 체액성 면역에 대한 연구는 많은 규명과 진전이 있어 왔으나 세포성 면역에 대한 기전이나 특성에 대한 연구는 미미한 상태이다. 저자들은 이와같은 사정을 감안하여 마우스를 실험 모델로 할 때에 요구되는 참고 자료와 기본 정보를 구하고자 하였다. 즉, 간흡충에 대한 세포 면역학적인 일부의 특성을 알아 보았으며, 특히 비장 림프구 아형에 대한 phenotype의 특성을 알아 본 결과 다음과 같은 성적을 얻었다. 간흡충의 조항원을 면역증강제와 함께 복강 투여한 후 기간 별로 비장 림프구의 아형 특성을 flow cytometry로 측정한 결과, CD2, CD5 그리고 CD8는 투여 초기에는 감소하다가 투여 7주 후에 다시 증가하는 현상을 보였으며, CD4의 경우 투여 초기에는 약간 증가하다가 투여 7주 후에 다시 증가하는 성적을 보였다.

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