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Effect of Challenge Number of *Clonorchis sinensis* Metacercaria on Transfer of Immunity in Hamsters*

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=초록=

肝吸蟲의 移入免疫을 받은 햄스터의 肝吸蟲 感染에 있어 被囊幼蟲數가 미치는 影響

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移入免疫을 받은 햄스터에서 challenge 感染시킬 때 被囊幼蟲數가 肝吸蟲의 worm burden에 미치는 影響을 관찰하기 위해 肝吸蟲 成蟲의 代謝産物과 Freund's incomplete adjuvant의 混合物을 1주 간격 2회 感作시킨 donor 햄스터의 腹腔細胞와 血淸을 recipient 햄스터의 腹腔內에 주입하였다. 7일 후에 肝吸蟲 被囊幼蟲 10, 20, 30, 40 및 50마리씩 challenge 經口 感染 시킨 다음 50일에 屠殺하여 免疫細胞 수와 worm burden을 決定하였다.

感染 후 EPG는 challenge 感作群보다 感染 對照群에서 일찍 나타났으며 모두 점차 증가 되었다가 40일 전후에 最高値를 나타내었다.

蟲體 回收率은 被囊幼蟲 10 및 20마리 感染시킨 感作群과 對照群 사이에 유의한 차이를 認定할 수 있었으나 被囊幼蟲 30, 40 및 50마리 感染시킨 感作群과 對照群에서는 유의한 차이를 인정할 수 없었다.

Plaque 형성세포는 被囊幼蟲 20마리 이상 感染시킨 感作群에서 검출되었으나 對照群에서는 檢出되지 않았다.

이상의 成績으로 golden 햄스터의 適切한 肝細胞 被囊幼蟲 challenge감염 수는 약 20마리임을 알 수 있었다.

Key Words: Challenge number, Transfer of immunity, *Clonorchis sinensis*

Introduction

Many experiments on the transfer of immunity against the infection of *Clonorchis sinensis* have been perrformed (Choi *et al.*, 1987; Choi and Park, 1987; Lim *et al.*, 1987; Cho *et al.*, 1988; Choi *et al.*, 1990; Kim *et al.*, 1994). Choi *et al.*(1987) showed that there was no effective transfer of immunity against *C. sinensis* in BALB/c mice and golden hamsters when the intraperitoneal(IP) injection of peritoneal cells(PC) obtained from the donor which were injected via IP or the footpads with the admixture of the metabolic products with Freund's incomplete adjuvant. However,

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Choi and Park(1987) demonstrated a successful transfer of immunity against *C. sinensis* in hamsters, when the recipient hamsters were injected IP with PC and serum from strongly sensitized donor.

Choi *et al.*(1990) used the number of 20 metacercariae of *C. sinensis* in the challenge infection of the recipient hamster, and significant reduction was found in worm burden, and the effective transfer of immunity was confirmed in the hamster.

However, there was no significant difference in the worm burden of *C. sinensis* between the recipient hamster and control one, when 50 metacercariae were challenged.

Moon *et al.*,(1994) carried out the study on the effect of necropsy days after challenge infection in hamsters in which plaque forming cells(PFC) were observed, and reported that the appropriate necropsy day of golden hamsters was around the 50th day after challenge infection. The object of this study is to determine the appropriate challenge number of *C. sinensis* metacercaria on the transfer of immunity in hamsters.

Materials and Methods

1. Animals

Male golden hamsters 6-8 months of age were used for immunization and passive transfer. The stock colony had been tested for histocompatibility between members by reciprocal transplantation of skin grafts.

2. Adult worm of liver fluke

The metacercariae of *C. sinensis* were collected from *Pnathopogon atromaculatus* caught in the river Chondo, Chongdo county, Kyungpook Province, Korea. About 300 metacercariae were given orally to rabbits. In three months after experimental infection, the adult *C. sinensis* in the biliary passage of the liver were collected. The fluke were washed in 3 times with physiological saline for 2 hours. The adult *C. sinensis* were stored in a refrigerator until the next experiments.

3. Preparation of metabolic products of adult *C. sinensis*

As described by Sun(1969), each adult fluke was cultured in a plastic tube(Falcon, U.S.A.) containing 0.5ml of M-199 for 5 days under the condition of 100% relative humidity and 5% CO_2 concentration. Powdered medium-199(Gibco, U.S.A.) was dissolved in 500ml of distilled water and penicillin-streptomycin liquid(Gibco, U.S.A.) was made by addition of penicillin 50 units and streptomycin 50ug per ml of M-199. The pH of the medium was adjusted to alkaline by the addition of 7% sodium bicarbonate.

4. Immune sensitization of donor hamsters

The donor hamsters were divided into 2 groups. Group 1 was sensitized by injecting 0.5ml of the admixture of the metabolic products of adult *C. sinensis* and Freund's incomplete adjuvant into footpads at two weeks' interval. Group II was the non-sensitized control group.

5. Collection of peritoneal cells and immune sera

The days after the last immunization, the peritoneal cells(PC) and immune serum were collected from donor hamsters. In order to collect PC, after irrigation of the abdominal cavity of the hamster with 5.0 ml of M-199, the PC were collected with a syringe. Determination of viable PC was done by the trypan blue exclusion method(Kruse and Patterson, 1973).

6. Passive sensitization of recipient hamsters

Recipient hamsters were injected intraperitoneally (IP) with 5×10^5 PC and 0.5ml of serum from group I donor hamsters. The control hamsters of Group 5, 6, 7 and 8 were injected IP with 5×10^5 PC and 0.5 ml of serum from group II non-sensitized donors(Table 1).

7. Challenge infection of passively sensitized recipient hamsters

Seven days after the passive sensitization, recipient hamsters were divided into 5 groups. Group 1 were challenged with 10 metacercariae, group 2 were challenged with 20, group 3 challenged with 30, group 4 with 40, and group 5 with 50 metacercariae of *C. sinensis*. The 5 groups of unsensitized control hamsters were infected with the same numbers of the metacercariae of *C. sinensis*, respectively.

8. Egg count and worm burdens

Fifteen days after challenge infection, stool exami-

Table 1. Challenge numbers of *Clonorchis sinensis* metacercariae on transfer of immunity against *C. sinensis* in golden hamsters

Donor Hamsters			Recipient Hamsters			
Group	No.	Sensitization methods	Group	No.	Primary sensitization	No. of metacercariae in Challenge infection
I	8	0.5 ml of metabolites and adjuvant mixture, injected two weeks interval	1	3	5×10^5 PC[a] and 0.5ml of serum from group I sensitized donor hamster injected IP[b]	10
			2	3	〃	20
			3	3	〃	30
			4	3	〃	40
			5	3	〃	50
II	8	control	1	3	5×10^5 PC and 0.5ml of serum from group II control donor hamster injected IP	10
			2	3	〃	20
			3	3	〃	30
			4	3	〃	40
			5	3	〃	50

[a] Pc means peritoneal cell. [b] IP means intraperitoneal

nation was performed by formalin-ether sedimentation technique(Ritchie, 1948) to demonstrate the eggs of *C. sinensis*. Once the eggs were demonstrated by the sedimentation technique, Stoll's eggs counting method (1923) was employed to determine the number of eggs per gram of feces(EPG) to the 50th day of challenge infection. Both challenged recipient and control hamsters were killed on the 50th day, and the worm burdens were determined by pressing the livers, gall bladder and bile ducts between two large slide glasses(9×12cm).

9. Indirect Jerne plaque assay

Bactoagar(Difco, U.S.A) was dissolved into 0.7% in M-199 solution. In order to eliminate the anti-complementary effect, 0.1ml of 10% DEAE dextran(Pharmacia Fine Chemicals, Sweden) was added to every 20ml of the agar solution. 1ml of packed SRBC was added a drop at a time into a beaker containing 7.0 ml of cacodylate buffer supplemented with 20ml of TNP for 10 minutes while stirring slowly with a magnetic bar. After concentration of the suspension at 2,000 rpm for 10 minutes, the precipitated SRBC were washed in two times with 35ml of MBB containing 7.3mg glycyl-glycin at 2,000 rpm for 20 minutes. The resulting precipitate was diluted 15-fold with M-199. Forty days after infection with 50 metacercariae of *C. sinensis*, the hamsters were bled and the serum was separated. The serum was treated with saturated ammonium sulfate. The mixture was centrifuged at 3,000 rpm for 3 minutes and the precipitate was recovered. The precipitate was dissolved in distilled water and dialysed overnight against phosphate buffered sa line(PBS, pH 7.2) at 4℃. The dialysed solution was mixed with an equal volume of Freud's incomplete adjuvant. The emulsion was injected subcutaneously to a rabbit and boosted 3 weeks after the primary injection. One week after the last boost, blood was collected from the ear vein. The spleen was separated and placed in a plastic tissue culture dish(Oxnard, USA) containing 5ml of ice-cold M-199. The spleen was placed on a stainless steel screen(100 mesh) in plastic petri dish(13×100mm) containing 5ml of cold M-199 and teased gently with the rubber policeperson(Difco, U.S.A). The cell suspension was diluted 4-fold with the M-199 before assay. The slides precoated with 0.1% agarose were placed on the slide warmer(Precision Scientific) adjusted at 42-45℃. Commercial guinea pig complement preabsorbed were used to prevent nonspecific lysis of SRBC. Lyophilized guinea pig complement(Gibco, U.S.A) was dissolved and diluted 10-fold in M-199. A assay(Jerne and Nordin, 1963; Zaleski, 1981) modified by Choi and Eun(1985) was employed. Seven percent agar solution in M-199 and culture tubes wre placed in a 45℃ water bath. 0.3ml of agar solution, 0.05ml of conjugated SRBC

suspension and 0.05ml of SC suspension were added and mixed competely to the prewarmed tube. The mixture was poured on the prewarmed slide. After jellification, the slides were placed agar side down on the specially-made complement tray and the space between the bottom of the tray and slides filled with M-199. The slides were incubated at 37℃, 5% CO_2 Concentration and 100% relative humidity for 60 minutes. After the initial incubation, the medium containing 10-fold diluted guniea pig complement and 300 times diluted rabbit anti-hamster IgG anti-sera was injected into the space and the slides incubated for an additional 60 minutes under the same conditions. The slides were then dried for 20 minutes, washed in distilled water and dried again as described by Fuji *et al.*(1971). The plaques formed on the slides were counted.

Results

Table 2 shows the proportion of viable and dead cells per ml of PC from donor hamsters by trypan blue exclusion technique. The average rates of viable PC for sensitized and control donor hamsters were 91.8 and 90.2% respectively, and the difference was not significant at 5% level. The effect of challenge number of the metacercariae on the egg production of *C. sinensis* between sensitized recipient and control hamsters from the 16th to the 50th day after challenge infection is presented in Table 3. The eggs of *C. sinensis* were detected for the first time on the 16th day of challenge infection in the all control groups, and on the 17th day of infection in the sensitized recipient groups, except for the 3rd recipient and control groups. The EPG of pooled feces in the recipient and control groups increased step by step until the 33rd day, and then irregularly increased from the 36th to the 50th day after challenge infection. There was a significant difference in EPG of pooled feces between the 2nd, 3rd recipient and those of control groups, but no significant difference in the 1st, 4th, 5th recipient and control groups. Table 4 summarizes the effect of challenge number of the metacercaria on the worm burdens of *C. sinensis* in the recipient and control hamsters on the 50th day at necropsy. In general, the mean recovery number of *C. sinensis* in the recipient was less than those of the control hamsters. The number of recovered *C. sinensis* in the recipient group 1 was 5.0 and that of control group 1 was 6.3 per hamster. The number in the recipient group 2 was 10.3 and in the control

Table 2. Rate of viable and dead cells of PC suspensions of donor hamsters in medium-199 by trypan blue exclusion technique

Group	Total No. of cells($X10^5$)	Mean No. viable cells($X10^5$)	Rate of viable cells(%)
sensitized donor hamsters	2.81	2.58	91.8
control donor hamsters	2.65	2.39	90.2

Table 3. Effect of challenge number of metacercariae on egg production of *C. sinensis* between sensitized recipient and control hamsters from the 16th to the 50th day after challenge infection.

Group		EPG from the 16th to the 50th day after challenge												
		17	18	20	23	26	29	32	35	38	41	44	47	50
Recipient	1	−[a)]	2	8	12	32	48	144	128	312	224	184	124	268
Control	1	+[b)]	4	10	16	40	76	158	234	240	210	274	238	220
Recipient	2	−	6	8	28	88	144	180	280	272	180	172	152	274
Control	2	+	8	14	34	76	170	198	256	290	200	228	318	270
Recipient	3	+	10	20	62	108	156	216	336	270	256	228	296	272
Control	3	+	12	26	78	128	186	250	288	340	416	266	240	360
Recipient	4	+	4	8	70	124	232	292	380	398	256	232	310	324
Control	4	+	10	28	108	136	210	324	260	278	370	326	290	308
Recipient	5	−	5	8	33	57	234	165	155	246	206	308	287	336
Control	5	+	6	11	29	53	154	198	281	221	367	229	214	348

a) −means positive by formalin-ether sedimentation technique.
b) −means negative by formalin-ether sedimentation technique.

Table 4. Effect of challenge numbers of metacercariae on worm burden of *C. sinensis* between recipient and control hamsters on the 50th day at necropsy

Group		No. of metacercariae of challenge infection	Interval between challenging and necropsy	Adult worm recovered				Worm recovery rate(%)	p value between recipient and control groups
				H[a)]1	H2	H3	Mean		
Recipient	1	10	50	4	5	6	5.0	50.0	p>0.05
Control	1	10	50	5	7	7	6.3	63.0	
Recipient	2	20	50	9	11	11	10.3	51.5	p<0.05
Control	2	20	50	13	14	15	14.0	70.0	
Recipient	3	30	50	14	15	17	15.3	51.0	p<0.05
Control	3	30	50	18	18	21	19.0	64.3	
Recipient	4	40	50	19	20	21	20.0	50.0	p>0.05
Control	4	40	50	22	22	23	22.3	55.8	
Recipient	5	50	50	24	22	20	22.7	45.4	p>0.05
Control	5	50	50	17	23	22	23.3	46.6	

a) H means hamster.

Table 5. Viable SC and plaque forming cells in recipient and control hamsters on the 50th day after challenge infection.

Group		Spleen cells per ml of cell suspension			Plaque forming cells Per spleen(ea)			
		Total No. of cells($X10^6$)	Mean of viable cells($X10^6$)	viable cells(%)	H1	H2	H3	Mean
Recipient	1	1.48	1.34	90.5	100	–	800	600
control	1	1.41	1.26	89.9	0	0	0	0
Recipient	2	1.36	1.24	91.0	1,800	2,000	2,300	2,100
control	2	1.36	1.22	90.0	0	0	0	0
Recipient	3	1.42	1.27	89.0	2,400	3,000	2,700	2,700
control	3	1.38	1.27	92.0	0	0	0	0
Recipient	4	1.42	1.33	93.4	2,000	2,900	3,500	2,800
control	4	1.46	1.28	88.0	0	0	0	0
Recipient	5	1.37	1.27	92.0	2,600	3,100	2,100	2,600
control	5	1.39	1.25	89.9	0	0	0	0

group 2 was 14.0 per hamster. The number of flukes in the 5 recipient groups ranged from 5.0 to 22.7 per hamster and in those of the control groups ranged from 6.3 to 23.3 er hamster. However, a decreasing recovery rate was found by the intensity of challenge infection. The rates in the recipient groups were 50.0% in group 1, 51.5% in group 2, 51.0% in group 3, 50.0% in group 4 and 45.4% in group 5. A similar tendency of worm recovery rates was revealed in the control groups. There were significant differences in the worm recovery rates between the 2nd and 3rd recipient and control groups, but no significant differences in the 1st, 4th and 5th recipient and control groups. Table 5 summarizes viability of SC in indirect Jerne plaque assay and the number of plaque forming cells(PFC) per spleen in the recipient and control groups on the 50th day after challenge infection. The viability of SC in the recipient and control groups ranged from 88.0 to 92.0, with an average of 90.3%. The mean number of PFC per spleen in the recipient group 1 was 600, increased to 2,100 in the recipient group 2, and continued at almost the same levels in the recipient groups 3, 4 and 5. However, PFC was not found in the all control groups.

Discussion

Kobayashi(1912) reported that the growth of *C. sinensis* depended upon the size of the experimental host as well as the number of the flukes in the hosts. The degree of suitability of a laboratory animal as an experimental host of *C. sinensis* has been determined by the recovery rate and the development of the fluke in the biliary passages of the animal(Wykoff, 1958; Rhee and Seo, 1968). In which, the recovery rate of *C. sinensis* was greatly influenced by capacity of the biliary passage of the host animal. Choi and Park(1987) used 30 metacercariae of *C. sinensis* in challenging the recipient hamsters and reported that the sudden increase of EPG was observed between 28 and 31 days in the recipient

ɔups, whereas it was encountered between 40 and days in the control group. The sudden increase of ᵊG was thought to be expelled the flukes into the :estinal tract. They also reported that IP injection of ɔ and sera, not spleen cells and sera, from the donor msters immunized with the metabolic products of *sinensis* caused the transfer of immunity to the re-ɔient hamsters. In a study of determining the sus-ptibility of inbred golden hamsters(Mesocrietus au-tus) to experimental infection with *C. sinensis*, Chung ıd Choi(1988) reported that the recovery rate for the ıke was 57.9% and the appropriate number of *C. ıensis* metacercaria in the hamster was 20, although e number of metacercaria administered increased, a 'oportionate decrease in the recovery rate was found. ıoi *et al.*(1990) used for the first time 20 metacercariae *C. sinensis* in challenge infection of the recipient ımsters, and reported that significant reductions were und in the worm burdens in a group of hamster given $\times 10^5$ PC and 0.5ml of serum from the donors with injections of the metabolites and the adjuvant into otpads at 2 weeks interval. However, significant di-ɜrence was not encountered when the excessive nu-ber, 50 metacercariae was given(Kim *et al.*, 1994). highly suggested that the greater number of young ıkes residing in the narrow space of the biliary pa-ages might be pushed out naturally to the intestinal act with the growth of flukes. The thrust of flukes ay change the EPG patterns and the worm burdens experimental animals during the period of growth. is suggested that significant reductions in the worm ırdens were found in the recipient hamsters, when ıch was challenged with 20 or 30 metacercariae(Choi ıd Park, 1987; Choi *et al.*, 1990). The transfer of ımunity to the recipient normal animals was deter-ined tentatively by significant differences in EPG ıtterns, worm burdens and plaque forming cells per ıleen between immunized and non-immunized groups. ɔ, SC and other lymphoid cells have been widely used the transfer of immunity to the recipient animals, ıd it is most useful to inject experimental hosts IP. ıe large numbers of cells are required from strongly ımunized donors to be successful in most cases(Larsh ıd Weatherly, 1975). Passive transfer of protective ımunity with antiserum has not been successful, when Landsteiner and Chase(1942) stressed the necessity for lymphoid cells. It should be added that Hayashi *et al.*(1984) examined the passive transfer of protective immunity against *Brugia malayi* by serum and/or SC from vaccinated BALB/c mice to control mice. Resistance was observed by the worm recovery in the peritoneal cavity of mice. The larva was not found from vaccinated mice, whereas, some larvae were found alive in the control mice. They suggested that an antibody-dependent immunological enhancement(ADIE) may occur in the experiments. Therefore, recipient animals were injected IP with both the immune lymphoid cells and serum to lead ADIE(Choi and Park, 1987; Kwon *et al.*, 1987; Lim *et al.*, 1987; Cho *et al.*, 1988; Choi *et al.*, 1990; Kim et *al.*, 1994; Moon *et al.*, 1994). In the transfer of immunity against trematodes, Larsh and Race(1964) and Corba *et al.*(1971) reported successful transfer of immunity to *Fasciola hepatica* infection by lymphoid cells. Dodd and Nuallain(1969) showed that antirabbit lymphocyte serum revealed striking suppression of cellular responses in the rabbit infected with *F. hepatica*, and lymphoid cells considered to be effective against the fluke. Lang *et al.*(1967) and Armour and Dargie(1974) reported that PC from donor mice infected with *F. hepatica* successfully confered immunity on the recipients. However, Fravell *et al.*(1980) and Sirisinha *et al.*(1983) failed to show the transfer of immunity to *Opisthorchis viverrini* in the recipient ha msters. Choi and Lim(1986) have failed to demonstrate the transfer of immunity against *C. sinensis* in the recipient mice. Wakelin and Lloyd(1976) reported that transfer of both mesenteric lymph node cells(MLNC) and serum brought about a marked acceleration of worm expulsion in all cases, even where MLNC or serum given separately failed to transfer a significant degree of immunity. In fact, the species of laboratory animals, the methods of immunizing donors, and source of donor cells to be transfered to recipients may effect the transfer of immunity against *C. sinensis*. The golden hamster was found as a more susceptible experimental animal than the mouse(Choi *et al.*, 1987). The interstrain differences of mice in the ability to transfer immunity against *C. sinensis* were apparent(Kwon *et al.*, 1987; Choi and Park, 1987; Choi *et al.*, 1990). The metabolites of the fluke were a more potent immunogen than so-

matic constituents. Intracutaneous injection of the metabolite immunogens admixtured with Freund adjuvant into footpads was more potent in induction of the immunity than IP injection of the admixture. Moon *et al.*,(1994) carried out a study to determine the appropriate necropsy day in the passive transfer of immunity against *C. sinensis* in golden hamsters, 4 groups of the recipient and control hamsters were challenged with 20 metacercariae of *C. sinensis* and killed on the 50th, 70th, 90th and 110th day after challenge infection, and reported that the appropriate necropsy day is around the 50th day after challenge. The results of Moon *et al.*, (1994) reconfirmed previous experiments that the necropsy days of the recipient hamsters were selected about 50th day after challenge infection. There were no diminishing significant worm burdens between the recipient and control hamsters in the period from 50th to 65th day after challenge(Choi *et al.*, 1987; Choi and Park, 1987; Cho *et al.*, 1988; Kim *et al.*, 1994). To determine the appropriate challenge number of *C. sinensis* metacercaria, the recipient and control hamsters in the present study, were challenged with 10, 20, 30, 40, and 50 metacercariae of the fluke and killed on the 50th day after the challenge. The most important results obtained in this study is the finding that the eggs of the control hamsters appeared earlier than those of the recipient groups. There were significant differences in the worm burdens of *C. sinensis* between the recipient groups challenged with 20 and 30 metacercariae and the control groups. In addition, there were a small number of PC in the recipient groups challenged with more than 20 metacercariae of the fluke, but no PC in the group challenged ith 10 metacercariae and control groups. A part of these results were in agreement with the data reported by Chung and Choi (1988), Choi and Park(1987), Choi *et al.*(1990) and Kim *et al.*(1994). As would be expected, small numbers of PC were found in the all recipient hamsters. It is likely that the antigenicity of the metabolic and the somatic constituents of *C. sinensis* is so weak that it is possible to produce a small number of PC, e.g. a low degree of immunity, when the recipient hamsters were immunized with lymphoid cells and serum from the strongly immunized donors.

Summary

In order to determine the challenge number of *C. sinensis* metacercaria in the transfer of immunity against *C. sinensis* and worm burden in golden hamsters, the donor hamsters were immunized with 2 injections of the admixture of the metabolic products of *C. sinensis* and Freund's incomplete adjuvant into footpads at a 2 weeks interval. The peritoneal cells and sera of immunized and control donor hamsters were injected intraperitoneally to the recipient hamsters. Seven days after the sensitization, the recipient and control hamsters were challenged with 10, 20, 30, 40, and 50 metacercariae of *C. sinensis*, and killed on the 50th day after the challenge. The EPG appeared eariler in control hamsters than in the recipient groups, and increased step by step and then reached a peak around the 40th day after challenge infection. There was significant difference in the worm burden of *C. sinensis* between the 2 recipient groups challenged with 20 and 30 metacercariae and control groups, but no significant difference between the groups challenged with 10, 40, 50 metacercariae and control groups. there were small number of plaque forming cell in the recipient groups challenged with more than 20 metacercariae, but no PFC in the group challenged with 10 metacercariae and the control hamsters. These results indicate that the appropriate challenge number of *C. sinensis* metacercaria in the passive transfer of immunity in golden hamsters is estimated around 20.

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