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Establishment of *Schistosoma japonicum* calpain-specific mouse T cell hybridomas and identification of a T cell epitope that stimulates IFNγ production

Yoshio Osada[a,*], Takashi Kumagai[a], Mariko Hato[b], Takashi Suzuki[b], Mohamed El-Malky[b], Hiroko Asahi[c], Tamotsu Kanazawa[a], Nobuo Ohta[b]

[a] *Department of Parasitology and Tropical Public Health, The University of Occupational and Environmental Health, Japan, Kitakyushu 807-8555, Japan*
[b] *Department of Molecular Parasitology, Nagoya City University, Graduate School of Medical Sciences, Nagoya 467-8601, Japan*
[c] *Department of Parasitology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

Received 22 April 2004; received in revised form 14 October 2004; accepted 19 October 2004
Available online 8 December 2004

Abstract

Calpain is a calcium-dependent cystein protease, and the homologues of schistosome are known as one of vaccine candidate molecules against schistosomiasis. Here, we established two IL-2 producing T cell hybridoma cell lines specific for *Schistosoma japonicum* calpain, to identify T cell epitope(s) on the molecule. Overlapping 15mer oligopeptides of calpain were synthesized and tested for their stimulatory abilities to the hybridomas. As a result, epitopes recognized by the two hybridoma lines were the same: EQLKIYAQRC. Spleen cells from calpain multiple antigenic peptide (MAP)-immunized BALB/c mice produced IFNγ upon stimulation with MAP or soluble worm antigen preparation (SWAP). The identification of the T cell epitope to stimulate Th1 response will contribute to the proper design of synthetic vaccines, evaluation of their protective potentials and elucidation of protective mechanisms in murine experimental schistosomiasis.

Keywords: *Schistosoma japonicum*; Calpain; T cell epitope

1. Introduction

Schistosomiasis is one of the most prevalent parasitic diseases and is affecting 200 million people worldwide [1]. In order to control this disease, various control measures have been conducted for a long time. For instance, mass chemotherapy to control morbidity, snail control to reduce transmission, improvement of sanitary conditions to reduce influx of stool/urine eggs into water bodies. These efforts, however, succeeded in controlling or eliminating schistosomiasis only in some parts of endemic areas in the world; e.g., Japan, Caribbean islands and Tunissia [1]. One of the major difficulties in achieving successful control is rapid re-infection after chemotherapeutic treatment. For this reason, vaccine development is urgently needed. Until now, there have been reported many vaccine candidates; e.g., glutathione-*S*-transferase (GST) [2], triose phosphate isomerase [3], fatty-acid binding protein [4], myosin (IrV-5) [5], 22.6 kD antigen [6], glyceraldehyde-3P-dehydrogenase (G3PDH) [7], paramyosin [8–10] and calpain large subunit [11–15]. Among them, paramyosin [9] and calpain [12,15] are the molecules which have been reported to exhibit protective effects through Th1 responses. Especially, *Schistosoma mansoni* calpain has been identified as a target molecule of a protective Th1 cell clone [12].

Calpain large subunit is a cystein protease which contains four domains [11]. *Schistosoma japonicum* calpain ho-

* Corresponding author. Tel.: +81 93 691 7431; fax: +81 93 602 4488.
E-mail address: yosada@med.uoeh-u.ac.jp (Y. Osada).

0264-410X/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2004.10.042

mologue was cloned and its immune stimulatory potential was demonstrated by the presence of calpain-specific antibodies in the sera of schistosomiasis patients [14]. In this same report, we also demonstrated that the sera from light-infection cases showed stronger reactivities to recombinant calpain than the sera from moderate/high infection cases. Additionally, vaccination with recombinant *S. japonicum* calpain fragment (A.A. 220–376) partially protected BALB/c mice against cercarial challenge [15]. The protective mechanism is still unclear, but it is at least partially due to Th1-biased immune responses [15].

In this study we established T cell hybridoma cell lines specific for *S. japonicum* calpain. The hybridomas produced IL-2 in the presence of syngeneic antigen presenting cells (APC) and a recombinant GST-calpain fusion protein. A T cell epitope EQLKIYAQRC was identified by comparison of stimulatory activities of synthetic oligopeptides and the epitope was shown to have Th1 response-inducing activity. Potential protective activities of the sequence are also discussed.

2. Materials and methods

2.1. Maintenance of S. japonicum (Japanese Yamanashi strain) life cycle

Outbred ICR mice (Japan SLC, Inc., Hamamatsu, Japan) were infected with 30–40 cercariae through shaved abdomen percutaneously. Six weeks later, the infected mice were perfused with physiological saline containing 0.45% tri-sodium citrate to recover adult worms. The collected worms were washed with physiological saline and frozen at −70 °C. Granulomatous livers of infected mice were minced and homogenized in a warring blender. The liver homogenate was centrifuged at 200 × *g* for 3 min repeatedly to make the supernatant clear. The egg-containing pellet was poured into aged tap water and kept under light for 1 h. Miracidia were collected and used for infecting intermediate host snails (*Oncomelania hupensis nosophora*), which were collected in the Kofu basin, ex-endemic area of schistosomiasis japonica. Each snail was infected with 4–5 miracidia. Four to six months later, the infected snails were crushed and emerging cercariae were used for the next infection of mice.

2.2. Soluble worm antigen preparation (SWAP)

Frozen worms were dispersed in phosphate buffered saline (PBS, pH 7.4) by ultrasonic treatment. The homogenate was centrifuged at 40,000 × *g* for 20 min. The supernatant was then collected and its protein concentration was determined. This antigen preparation was kept at −70 °C until use.

2.3. Preparation of recombinant calpain and its deletion fragments

The section of *S. japonicum* calpain (A.A. 220–376) that has been reported to be protective [15] was introduced to BamHI-EcoRI site of pGEX-2TK vector (Amersham Biosciences UK, Ltd., Little Chalfont, UK). Expression of the recombinant protein was induced by 0.1 mM IPTG. Harvested cells from 200 mL culture were lyzed with 4 mL of BugBuster® Protein Extraction Reagent (EMD Biosciences, Inc., San Diego, CA) containing 5 units/mL of Benzonase® Nuclease (EMD Biosciences, Inc.) by vigorous shaking at room temperature for 20 min. Then the lysate was centrifuged at 25,000 × *g* for 20 min. An insoluble pellet was washed with PBS containing 1% Triton X-100 twice to remove membrane proteins. The washed pellet (inclusion body) was then solubilized with 4 mL of denaturing solution (8 M Urea and 5 mM DTT in PBS) by pipetting and vigorous shaking for 20 min. By the addition of 3-fold volumes of PBS containing 5 mM DTT, urea concentration of the solution was adjusted to 2 M. The GST-calpain fusion protein was purified by using Glutathione-Sepharose 4B® gel (Amersham). Alternatively, after the fusion protein was applied to the gel, the GST portion and calpain portion were separated with thrombin. By this treatment, only the calpain portion was eluted. The purified proteins were dialyzed in PBS to remove urea and DTT. Protein concentration was measured by the Protein Assay Kit® (Bio-Rad Laboratories, Hercules, CA). For immunization of mice to produce T cell hybridomas, the purified calpain was used. For cytokine production experiments (including hybridoma screening), GST-calpain fusion protein was used to stimulate cells. To exclude the possibility that hybridomas reacted to GST portion of the fusion protein, purified GST was simultaneously used in the same experiments. As for cytokine production assay using GST-calpain deletion fragments, inclusion body suspensions in PBS were used for stimulation of hybridomas because it was difficult to obtain the recombinant proteins as soluble forms in PBS.

2.4. Immunization of mice and culture of lymph node (LN) cells

Female BALB/c mice were immunized with 5 μg of purified recombinant calpain fragment in emulsified condition with Freund complete adjuvant (FCA, Difco Laboratories, Detroit, MI). The emulsion was injected into the four footpads and at the base of the tail intradermally. One week later axillary, inguinal and popliteal LNs were collected and pooled. LN cells were then cultured at 3×10^6/mL for 4 days in the presence of 2 μg/mL recombinant calpain fragment and 20 units/mL of recombinant human IL-2 (Roche Diagnostics, Mannheim, Germany). Then the cells were harvested and supplied for fusion.

2.5. Fusion and screening of T cell hybridomas

The cultured LN cells and BW5147.G.1.4 (ATCC No. TIB-48) thymoma cells were mixed at 2:1 ratio. One milliliter of 50% polyethylene glycol 1500 solution (Roche Diagnostics) was added to the cell pellet under continuous gentle shaking at 37 °C. FCS-free DMEM medium (Sigma-Aldrich,

St. Louis, MO) was further added and centrifuged at 250 × *g* for 5 min. The cells were re-suspended in DMEM supplemented with 10%FCS, 100U/mL penicillin and 100 μg/mL streptomycin. The final density of the thymoma cell was approximately 1×10^6/mL. One hundred microlitres of the suspension was poured onto 96-well microplates and cultured in the medium supplemented with HAT (Sigma-Aldrich) for 2 weeks. Normal thymocytes of BALB/c mice were added to the culture as feeder cells for hybridoma growth. If cell proliferation was observed in some wells, the cells were transferred to 24-well plates and further expanded. Screening of the cells was performed by IL-2 producing activity in the presence of syngeneic spleen cells and GST-calpain fusion protein. To deny the possibility that the cells react to GST portion of the fusion protein, recombinant GST was also used for stimulation in the screening step. Cloning of positive cells was performed by repeated limiting dilutions. Established clones were tested again for their reactivity. Their surface markers (CD3, CD4 and CD8) were examined by flow cytometry.

2.6. Cytokine production assay of T cell hybridoma

For screening and subsequent experiments, 1×10^5 T cell hybridoma cells and 2×10^5 syngeneic BALB/c spleen cells were co-cultured in the presence of antigens (5 μg/mL of GST-calpain, 50 μg/mL of GST, 25 μg/mL oligopeptides or 50 μg/mL suspension of inclusion bodies) in 200 μL of DMEM supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Twenty-four hours later, culture supernatants were collected and stored at −70 °C until measurement of IL-2. The ELISA Development Kit® for mouse IL-2 (Techne Corp., Minneapolis, MN) was used to measure the levels of IL-2 in the supernatants. In some experiments for multiple cytokine assays, cultures were performed in 0.5–1.0 mL under the same cell density conditions as above.

2.7. Synthesis of oligopeptides and MAP

Pentadecamer oligopeptides (for epitope screening) and multiple antigenic peptides (MAP) of the epitope sequence (for cytokine assay) were synthesized by Biologica Co., (Nagoya, Japan) and Nikka Techno Service Co., Ltd. (Hitachi, Japan), respectively. For the construction of MAP, 5 amino acids of N-terminus and C-terminus flanking the identified core epitope (EQLKIYAQRC) were included; i.e., the sequence was PQDLI-EQLKIYAQRC-CLMGC. The lyophilized powders of synthetic peptides and MAP were dissolved or suspended in PBS and then used for stimulation of T cell hybridomas or MAP-immunized mouse spleen cells.

2.8. Immunization of mice with MAP and cytokine production assay

Lyophilized MAP powder was suspended and homogenized in PBS by ultrasonic treatment because it was not soluble in PBS. MAP suspension (100 μg/mL) was mixed with an equal volume of FCA and emulsified. Two hundred microlitres of the emulsion (containing 10 μg of MAP) was injected into two sites of dorsal skin of BALB/c mice subcutaneously. Three weeks later, similarly prepared Freund incomplete adjuvant (FIA, Difco) emulsion of MAP was injected as a booster immunization. Three weeks after the booster immunization, the mice were used for the cytokine production experiments. Spleen cells (3×10^6) of the mice were cultured with antigens in RPMI 1640 supplemented with 10%FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM 2-mercaptoethanol in 0.5 mL culture. Concentrations of MAP, oligopeptides and SWAP used for stimulation were 20, 20 and 100 μg/mL, respectively. Forty-eight hours later, supernatant was collected and kept at −70 °C until use. Cytokines were measured using the Ready-Set-Go! Kit® (eBioscience, San Diego, CA) or the ELISA Development Kit® for mouse cytokines (Techne).

3. Results

3.1. Establishment of T cell hybridomas specific for S. japonicum calpain

Two lines of calpain-specific T cell hybridomas were established. The profiles of CD expression and cytokine production of them are summarized in Table 1a and b, respectively. When stimulated with GST-calpain fusion protein, both cell lines produced IL-2 in the presence of syngeneic spleen cells but did not produce IL-2 in the presence of non-syngeneic spleen cells (Table 1b). Both hybridomas did not produce detectable levels of IFNγ and produced a very little amount of IL-4 (Table 1b).

Table 1
Characteristics of calpain-specific T cell hybridomas

(a) Expression of CD molecules

Clone	CD3	CD4	CD8
BALB/SjCalp.1	+	+	−
BALB/SjCalp.71	+	−	−

(b) Cytokine production

Clone	APC	GST-calpain	IL-2	IFNγ	IL-4
BALB/SjCalp.1	BALB/c	−	<16	<16	<4
	BALB/c	+	1697 ± 71	<16	19 ± 2
	C3H/He	+	<16	NT[a]	NT
	C57BL/6	+	<16	NT	NT
BALB/SjCalp.71	BALB/c	−	<16	<16	<4
	BALB/c	+	1268 ± 125	<16	17 ± 2
	C3H/He	+	<16	NT	NT
	C57BL/6	+	<16	NT	NT

The hybridoma clones were stimulated in the presence of APC and GST-calpain (5 μg/mL). Representative data of similar experiments performed. Error values are S.D. of triplicate assays.

[a] NT: not tested. The units of cytokines are pg/mL.

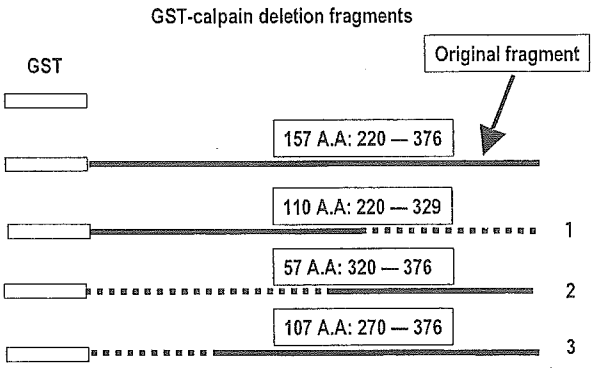

Fig. 1. Schematic presentation of GST-calpain (original fragment) and three deletion fragments (No. 1–3). Fragment No. 1 and No. 2 overlapped 10 amino acids. All fragments were expressed as fusion proteins with GST and used for cytokine assay without removal of GST portion.

3.2. Identification of a T cell epitope recognized by the T cell hybridomas

Firstly, we narrowed the region of the calpain fragment that contains T cell epitope(s). We produced three GST-calpain deletion fragments as shown in Fig. 1. Then the stimulatory activity of each fragment for IL-2 production was examined. As summarized in Table 2, only fragments 1 and 3 had activity to stimulate both hybridomas. We concluded that there must be epitopes in the central portion (A.A.270–329) of the calpain original fragment. Based on this result, we synthesized overlapping 15mer oligopeptides covering the central portion of the calpain fragment and stimulatory activities of each oligopeptides were examined. The oligopeptide No. 2 (PQDLIEQLKIYAQRC) and No. 3 (EQLKIYAQRCCLMGC) stimulated both T cell hybridomas to produce IL-2 (Fig. 2 and Table 3). Therefore, the core epitope of the calpain fragment recognized by the hybridomas was determined to be EQLKIYAQRC.

3.3. IFNγ producing activity of synthetic MAP-immunized mouse spleen cells

IFNγ is known to be a key cytokine in protective immunity against schistosome in mice [17–19]. As cytokine profiles of T cell hybridomas do not reflect those of original T cells [16], it is necessary to know the IFNγ producing potential

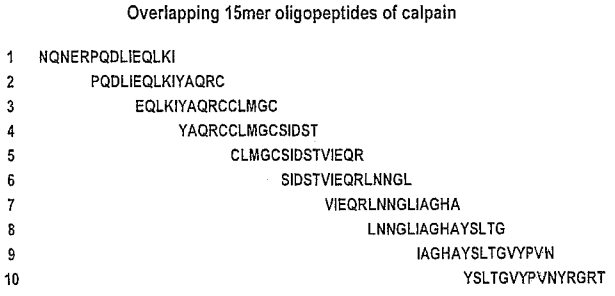

Fig. 2. Schematic presentation of synthetic 15mers covering the central portion (A.A. 270–329) of the original calpain fragment. All oligopeptides overlapped 10 amino acids with the following oligopeptides.

of the identified T cell epitope. Accordingly, we synthesized MAP, immunized BALB/c mice with it and examined IFNγ production by spleen cells from immunized mice. As shown in Table 4, spleen cells from the immunized mice produced IFNγ upon stimulation of the MAP. IFNγ production was also observed when the same cells were stimulated with the oligopeptide No. 2 or No. 3, which contains the core epitope sequence (Fig. 2, Table 4). Importantly, soluble worm antigen preparation (SWAP) stimulated the sensitized spleen cells to produce IFNγ. In addition, low levels of Th2 cytokine (IL-4 and IL-10) production were observed in the antigen-stimulated spleen cell culture supernatants.

4. Discussion

There are a large number of reports on vaccine-induced immunity in murine experimental schistosomiasis. Many of them focused on vaccine-induced immunity induced by attenuated cercariae. The crucial importance of IFNγ and Th1 immune responses have been shown in such reports on *S. mansoni* [17–19]. Likewise, the importance of IFNγ was suggested in protection against *S. japonicum* infection [20]. Also in human schistosomiasis mansoni, IFNγ was produced in a larger amount by PBMC in endemic normal individuals [21]. Therefore, it is meaningful to develop vaccines that exhibit their protective immunity through Th1 responses, in addition to developing antibody-based vaccines.

Calpain of *S. mansoni* was identified as a target molecule of protective Th1 clone of C57BL/6 mice [12]. In this context,

Table 2
IL-2 production by hybridoma cells upon stimulation with calpain deletion fragments

Clone	Soluble forms[a]		Inclusion body suspensions[b]			
	GST	GST-calpain	GST-calpain	GST-calpain deletion fragments		
				1	2	3
BALB/SjCalp.1	37 ± 37	>2000	961 ± 69	890 ± 132	20 ± 16	993 ± 170
BALB/SjCalp.71	77 ± 30	>2000	541 ± 45	468 ± 50	<16	465 ± 67

The unit of IL-2 is pg/mL. Each deletion fragment is shown in Fig. 1. Representative data of similar experiments performed. Error values are S.D. of triplicate assays.

[a] T cell hybridoma clones were stimulated with soluble forms of 50 and 5 μg/mL of GST and GST-calpain, respectively.

[b] As for inclusion bodies, 50 μg/mL suspensions were used for stimulation.

Table 3
IL-2 production by hybridoma cells upon stimulation with each oligopeptide

Clone	GST	GST-calpain	GST-calpain oligopeptides									
			1	2	3	4	5	6	7	8	9	10
BALB/SjCalp.1	22 ± 0	1600 ± 76	24 ± 8	>2000	1637 ± 124	23 ± 1	23 ± 16	<16	19 ± 4	23 ± 4	18 ± 2	25 ± 4
BALB/SjCalp.71	<16	1313 ± 119	<16	>2000	927 ± 73	<16	<16	<16	<16	24 ± 18	34 ± 20	<16

The unit of IL-2 is pg/mL. The sequence of each oligopeptide is shown in Fig. 2. T cell hybridoma clones were stimulated with 50, 5, and 25 μg/mL of GST, GST-calpain and oligopeptiodes, respectively. Representative data of similar experiments performed. Error values are S.D. of duplicate assays.

Table 4
Cytokine production by spleen cells from MAP-immunized BALB/c mice

Immunization	Cytokine	No Ag	MAP	Peptide 2	Peptide 3	SWAP
PBS + FCA/FIA	IFNγ	<78	<78	<78	<78	407 ± 241
	IL-4	11 ± 2	9 ± 2	8 ± 2	8 ± 2	20 ± 2
	IL-10	29 ± 13	<16	33 ± 17	16 ± 26	<16
MAP + FCA/FIA	IFNγ	<78	4903 ± 487	3064 ± 550	2676 ± 791	3807 ± 1431
	IL-4	<4	49 ± 1	59 ± 5	54 ± 2	54 ± 5
	IL-10	20 ± 20	46 ± 19	96 ± 33	44 ± 12	40 ± 25

The unit of cytokines are pg/mL. See Fig. 2 for sequences of peptide 2 and 3. The spleen cells were stimulated with MAP, oligopeptides (each 20 μg/mL) or SWAP (100 μg/mL). Representative data of similar experiments performed. Error values are S.D. of triplicate assays.

we cloned an *S. japonicum* homologue of calpain large subunit [14] and demonstrated that a recombinant calpain fragment induced Th1-biased protective immunity in BALB/c mice in our previous studies [15]. Based on these results, here we tried to identify T cell epitope(s) of the protective calpain fragment of *S. japonicum* by the establishment of T cell hybridomas from BALB/c mice. Then we confirmed IFNγ producing potential of the epitope.

We immunized BALB/c mice with purified recombinant calpain fragment and established two lines of T cell hybridoma. Both lines produced IL-2 upon stimulation with GST-calpain fusion protein but did not produce IL-2 upon stimulation with GST (Tables 2 and 3), indicating that these hybridomas were specific for the calpain portion of the fusion protein. Then, their CD expression and cytokine profiles were briefly tested. As expected, both were positive in CD3 molecule (Table 1a). As for CD4/CD8 molecules, BALB/SjCalp.1 was CD4 -positive and BALB/SjCalp.71 was double negative (Table 1a). According to the brief cytokine production assay, only IL-2 was prominently produced by both hybridomas (Table 1b). IFNγ was not produced at a detectable level and IL-4 was produced at a very little amount (Table 1b). As these results may be due to the timing of supernatant collection that is appropriate for IL-2 assay (24 h), we measured IFNγ levels at 48 h. However, we did not observe the increase of IFNγ levels in the 48 h supernatants (data not shown). At all events, prediction of cytokine profiles of original T cells by those of T cell hybridomas is not possible. The reason is that fusion with BW5147.G.1.4 thymoma cells affects cytokine profiles of original T cells [16].

To narrow the region of epitope(s), we constructed deletion fragments of the calpain fused to GST (Fig. 1). As it was very difficult to solubilize the fusion proteins of GST-calpain deletion fragments in PBS, we used inclusion body suspensions (mostly constituted with recombinant proteins) of each recombinant *E. coli* lysate for stimulation of the T cell hybridomas. As shown in Table 2, only fragment No. 2 failed to stimulate the T cell hybridomas. These results indicated that epitope(s) should be located at the central portion (Fig. 1, A.A.270–329) of the original calpain fragment. The lower levels of IL-2 when stimulated with inclusion body suspensions of GST-calpain were probably due to the antigen conditions different from those of soluble form of the same antigen. In addition, the amounts of IL-2 produced by these T cell hybridomas usually fluctuated very much between independent experiments, even cultured in the same condition. We do not know the reason; however, this is probably because of the unstable cell conditions of the hybridomas.

Based on the result of Fig. 2, 10-amino acid-overlapping 15mer oligopeptides were synthesized covering the full length of the central portion (Fig. 2). Among them, only No. 2 and No. 3 oligopeptides stimulated both hybridomas to produce IL-2 (Table 3). This means that the epitope recognized by the two hybridomas were common; i.e., EQLKIYAQRC, A.A.280–289 (Fig. 2, Table 3).

As noted above, the cytokine profiles of T cell hybridomas do not reflect the profiles of the original T cells [16]. For this reason, it was necessary to check IFNγ production by lymphocytes of calpain-immunized or MAP-immunized mice, to determine the Th1 response-inducing potential of the epitope. As shown in Table 4, spleen cells from synthetic MAP-immunized BALB/c mice produced IFNγ upon stimulation with MAP (immunogen) or oligopeptides No. 2 and 3 containing the epitope sequence EQLKIYAQRC. Additionally, the spleen cells produced IFNγ when stimulated with SWAP (Table 4), implying that calpain molecules contained in the SWAP sample stimulated MAP-sensitized T cells. This result showed high possibility that if mice were immunized with MAP, IFNγ would be produced in vivo following challenge infection. As IFNγ is known to be a crucial cytokine

for protective immunity in mice [17,19], it is expected that immunized mice would be protected from challenge infection of normal cercariae. However, the levels of vaccine-induced protection against schistosome do not depend on IFNγ production levels only, but rather depend on the balance of Th1/Th2 cytokines [20,22]. Here, Th2 cytokines (IL-4 and IL-10) were also produced when the sensitized spleen cells were cultured with the immunogen (MAP) or SWAP, but their levels were relatively low (Table 4). This is consistent with our previous report [15], which demonstrated *S. japonicum* calpain preferentially induced Th1-biased immune response.

Yang et al. [23] reported inefficacy of multi-epitope vaccination to protect mice against challenge infection of *S. mansoni* although their vaccine constructs contained T cell epitope of calpain [12] in C57BL/6 mice. It might be necessary to design appropriate immunization protocols to induce protective Th1 responses, in addition to identification of Th1-inducing T cell epitopes.

In conclusion, here we have identified a T cell epitope of *S. japonicum* calpain that can induce Th1 response in BALB/c mice. Further experimental studies to improve adjuvant formulation and immunization protocols in mice, as previously reported with calpain [24–27], will contribute to vaccine development against schistosomiasis.

Acknowledgements

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 15390137), the Ministry of Health, Labor and Welfare of Japan (H15-Shinko-8) and the US-Japan Cooperative Medical Science Program.

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Nagoya Med. J. (2005) 47, 111–122

STRAIN DIFFERENCES OF THE IMMUNOSTIMULATORY EFFECT OF CPG IN OVA-SENSITIZED MICE

NOBUAKI OHNO[1], MOTOHIKO SUZUKI[1], TAMAMI MATUMOTO[1], TAKU OHASHI[1], SHINGO MURAKAMI[1] AND NOBUO OHTA[2]

[1]*Department of Neurotology, and* [2]*Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

(*Accepted for publication January 13, 2005*)

SUMMARY

Immunotherapy using adequate adjuvants to enhance immunogenicity and reduce allergenicity has been ideal for the treatment of allergic disease. Recently, immunostimulatory oligodeoxynucleotides containing CpG motifs have been shown as a strong Th 1 inducing adjuvant and demonstrated that co-administration of antigen results in modulation of allergic Th 2 immune response in murine models. In contrast to the effectiveness, no report have been reported the difference of the immunomodulatory effect between BALB/c and C 57 BL/6 mice. Our investigations were conducted to compare the effectiveness of CpG immunization in Th 2 sensitized those mice. At first, we examined IL-12 p 40 production from nasal lymphocytes of non-immunized mice stimulated with CpG in vitro. We found that nasal lymphocytes from BALB/c mice produced higher levels of IL-12 p 40 than those cells from C 57 BL/6 mice. Next, both strains of mice were sensitized with ovalbumin (OVA) intraperiotoneally and then immunized with CpG. OVA-specific IgE, IgG 1, and IgG 2 a antibody responses, Th cytokines from nasal lymphocytes were investigated. OVA plus CpG co-administration induced Th 1 response and reduced Th 2 response in both strains of mice. However, the immunomodulatory effect of CpG in BALB/c mice was higher than that in C 57 BL/6 mice. These results

大野伸晃，鈴木元彦，松本珠美，大橋　卓，村上信五，太田伸生

Address for Correspondence: Nobuaki Ohno, Department of Neurotology, Nagoya City University Graduate School of Medical Science, Kawasumi 1 Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan.

Tel: 052-853-8256, Fax: 052-851-5300, E-mail: nobuo@med.nagoya-cu.ac.jp

suggest that the effectiveness of CpG for allergic rhinitis was strain dependent and might be useful to clinical application of CpG based immunotherapy for humans.

Key words: CpG motifs, strain, ovalbumin, rhinitis, mouse

INTRODUCTION

Allergic rhinitis, one of the most prevalent allergic disease characterized by antigen-specific IgE production and eosinophilia, has increased in recent years. Previous studies have indicated that the dominance of Th 2-cytokines (IL-4, IL-5 and IL-13) lead to the clinical manifestations (1-4). On the other hand, skewing from Th 2 toward Th 1-response in immune microenvironment was needed to suppress allergic inflammation (5, 6). Specific immunotherapy has been the most effective treatment for desensitization of allergen and chosen for more than 90 years (7). Although effective, this treatment has fallen out of favor because of the risk of allergen-induced anaphylatoxic shock and frequent injection for several years.

Recently, the strong ability to skew immune response toward Th 1 has been demonstrated with bacterial DNA (8, 9). Such the treatment drives activation of CD 4+ Tcells producing IFNs and cytotoxic CD 8+ Tcells. Furthermore, oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs have been shown to activate macrophages and dendritic cells to secrete IL-12 and induce IFN-γ secreting Th 1 cells (10-13). Many reports have indicated that CpG based therapies can prevent Th 2 biased immune responses and/or attenuate allergic hypersensitivity responses in animal models (14-17). In those reports, coadministration with CpG and antigens induced stronger Th 1 immune response with high levels of antibody and cytokine production than CpG alone. More recently, antigen in the conjugated forms with CpG was 100-fold more efficient in regulating airway eosinophilia and inducing Th 1 differenciation in vitro (18-20). Indeed, clinical trials have demonstrated in ragweed-sensitive patient. Treatment with ragweed allergen (Amb a 1) -CpG conjugate by subcutaneous injection before pollen season decreased nasal symptom and nasal eosinophilia during the second ragweed season (21). However, it is still controvertible about diversities of the effectiveness determined by the genetic background.

In this report, we compared the efficiency of CpG between the two different mouse strains, BALB/c and C 57 BL/6. CpG and OVA were administrated intranasally or intradermally in OVA-presensitized mice. To evaluate CpG-induced immune response, antigen spe-

cific antibody levels including IgE in serum and antigen-driven cytokines production including IL-4, IL-5 and IFN-γ from nasal lymphocytes were measured. Together with those results, we discuss the probable differences in efficacy of CpG in different genetic background. This might be applicable for future clinical use of CpG for treatment and/or prophylactics of allergic diseases in humans.

MATERIALS AND METHODS

Animals and reagents

Female BALB/c and C 57 BL/6 mice were purchased from Charles River of Japan (Tokyo, Japan). The mice were used in these experiments at 7 weeks of age. Mice were maintained in our animal facility. Chicken Ovalbumin (OVA) was purchased from Sigma (St. Louis, USA) and CpG oligodeoxynucleotide containing two CpG motifs (TCCATGACGTTCCTGACGTT) and non-CpG which CpG motifs were modified (TCCATGAGCTTCCTGAGCTT) were synthesized by Rikaken Co. (Nagoya, Japan). This experiment was approved by the Ethycal Committee for Animal Rights of Nagoya City University Medical School (Permission #H 15-15)

Immunization of mice

Mice were sensitized intraperitoneally with 10 μg of OVA (Sigma) dissolved in 200 ml saline containing 2 mg of aluminum hydroxide (alum) two times at two weeks interval. Two weeks after last sensitization, mice were immunized intranasally with OVA alone, OVA+CpG 5 μg, OVA+CpG 50 μg and OVA+non CpG 50 μg. One day before and two weeks after the immunization, blood samples were collected from tail veins.

OVA-specific IgE

OVA-specific IgE was determined by sandwich ELISA as previously described (22). Briefly, ELISA plates (Corning,USA) were coated with 5 μg/ml rat anti-mouse IgE (clone LO-ME-3, Biosource, USA) in 0.05 M carbonate-bicarbonate buffer pH 9.6 overnight at 4 ℃. These plates were washed with PBS containing 0.05% Tween 20 and were blocked by PBS containing 10% FBS and 0.3% Tween 20 for 2 hours at 37℃. Then, pooled serum samples were incubated at 1/6 dilution for 2 hours at 37℃. After washing the plate with PBS containing 0.05% Tween 20, biotinylated OVA (1 μg/ml) were plated and incubated for 2 hours

at 37℃. After washing, the plates were incubated with extraavidin-peroxidase conjugate (Sigma) at 1/1000 dilution for 1 h at 37℃. Finally, tetramethylbenzidine substrate was added to the plates and the reaction was stopped by 5 % phosphoric acid. The absorbance at 450 nm was measured with a microplate reader (Biorad, USA).

OVA-specific IgG 1 and IgG 2 a titers

OVA specific IgG 1 and IgG 2 a were analyzed by indirection ELISA as previously described (22). Briefly, ELISA plates (Corning) were coated with 20 μg/ml OVA overnight at 4 ℃ and blocked for 2 hours. Next, 1/100 diluted serum samples were added for 2 hours at 37℃. Then, plates were incubated with peroxidase-labelled goat anti-mouse IgG 1 at a 1/10000 dilution and IgG 2 a at a 1/4000 dilution for 1 h. Finally, substrate and stop buffer was added. The absorbance at 450 nm was measured.

In vitro stimulation of nasal lymphocytes

Nasal lymphocytes were prepared by enzyme extraction with collagenase as previousely described (23). In first experiment, the lymphoid cells (2×10^6 cells/ml) were cultured with CpG at serial dilutions for 48 h at 37℃ in humidified air containing 5 % CO_2 in RPMI 1640 medium (Sigma) supplemented 10% FBS, and 100 unit/ml and 100 μg/ml penicillin G/streptomysin (Sigma) in 96 well flat bottom plates. In next experiment, those cells were cultured with OVA (30 μg/ml) or without OVA for 72 h at 37℃. Cell supernatants were collected and stored at -80℃ until investigation.

Cytokine analysis

IL-5 and IFN-γ levels were measured by captured ELISA as previously described (24). Briefly, ELISA plates were coated with 50 μl of capture mAb (rat anti-mouse IL-5 and IFN-γ from paharmingen, USA) in carbonate buffer overnight at 4 ℃ and blocked for 2 h. Next, the culture supernatant or recombinant standards at serial dilutions were added for 2 h at 37℃. Then, plates were incubated with biotinylated rat antimouse cytokine mAb (IL-5 and IFN-γ; Pharmingen) for 1 h. Finally, substrate and stop buffer was added. The absorbance at 450 nm was measured. The detection limits of IL-5 and IFN-γ were 15 pg/ml, and 20 pg/ml, respectively. IL-4 and IL-12 levels were measured by captured ELISA using IL-4 and IL-12 p 40 OPTEIA™ system (BD biosciences, USA).

RESULTS

IL-12 production by nasal lymphocytes stimulated with CpG

Nasal lymphocytes from naive BALB/c mice and C 57 BL/6 mice were cultured with CpG alone in serial dilution. Both strains of mice produced a significant ($p<0.05$) amount of IL-12 after stimulation with 5 and 50 μgCpG (Fig. 1). However, nasal lymphocytes from BALB/c mice showed higher level of IL-12 compared to those from C 57 BL/6 mice (Fig. 1).

Antigen specific IgE production following intranasal immunization with CpG

After intranasal immunization with OVA plus CpG 50 μg, both strains of mice showed a significant ($p<0.05$) decrease in IgE level compared with those immunized with OVA alone or OVA plus non CpG. Mice treated with OVA plus 5 μg CpG produced an increased amount of OVA-specific IgE, which were comparable to those immunized with OVA alone. In the two mouse strains, BALB/c displayed stronger suppression than C 57 BL/6 significantly when CpG was added at the dose of 50 μg/ml (Fig. 2).

Antigen specific IgG production after intranasal immunization with CpG

In these experiments, we investigated effects of CpG on the production of Th 2-controlled Ig (IgG 1) and Th 1-controlled Ig (IgG 2 a). Production of IgG 1 was not significantly ($p<0.05$) suppressed in both mice after intranasal administration of OVA plus 50 μgCpG compared to mice with OVA alone (Fig. 3). In contrast, we observed significantly ($p<0.05$) increase in IgG 2 a production in both mouse strains treated with OVA plus 50 μgCpG compared to mice with OVA alone or OVA plus non CpG. BALB/c mice showed more apparent elevation of IgG 2 a production following the immunization with CpG than the case of C 57 BL/6 (Fig. 4).

Cytokine production by nasal lymphocytes in vitro

Nasal lymphocytes from OVA-sensitized mice produced detectable levels of IL-4 and IL-5 by restimulation of OVA. The amounts of cytokines tested were comparable between BALB/c and C 57 BL/6 mice (Fig. 5, A, B, C and D). In contrast to the case of OVA alone, nasal lymphocytes from OVA plus 50 μgCpG immunized mice showed significantly decreased amounts of those cytokines and increased amounts of IFN-γ (Fig. 5, E and F) ($p<0.05$). BALB/c mice showed stronger downregulation of Th 2 and upregulation of Th 1 than C 57 BL

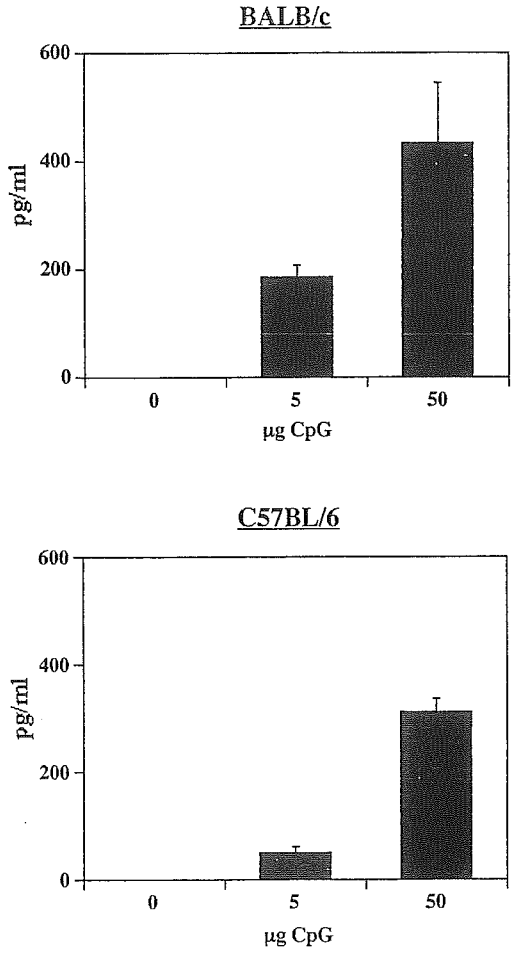

FIG. 1. IL-12 production by nasal lymphocytes from native BALB/c and C 57 BL/6 mice. Nasal lymphocytes were collected from two noses of mice and cultured for 48 h with 5 μg, 50 μg CpG or supplemented medium alone as a negative control. The supernatants were assayed by ELISA. Results show the mean±SEM from five wells. Detection limit for IL-12 p 40 was 25 pg/ml.

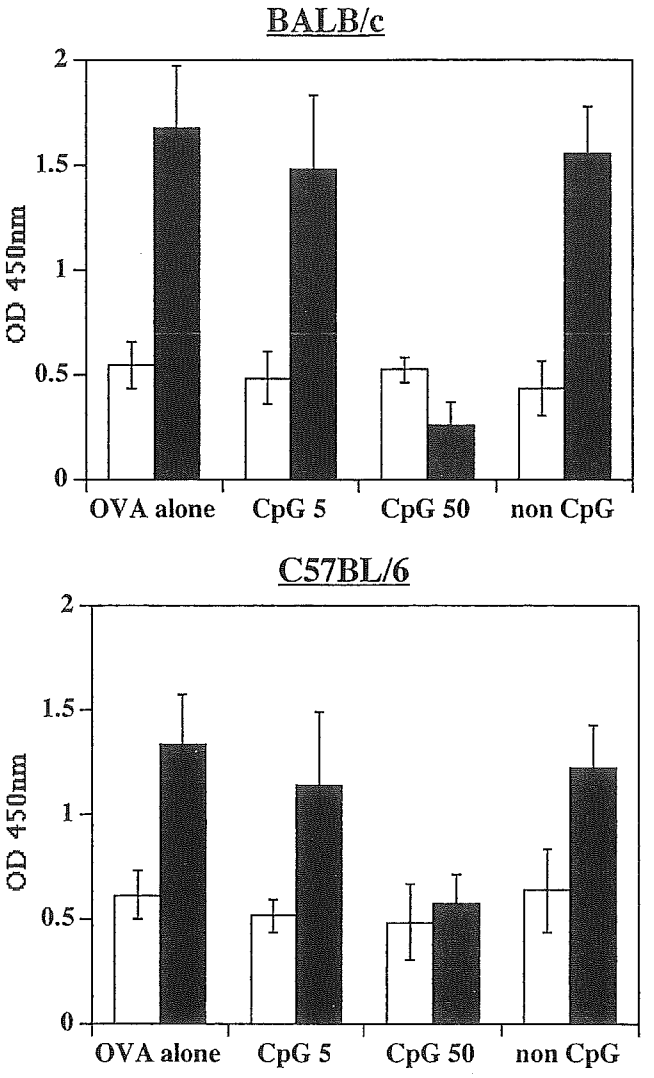

FIG. 2. Levels of serum OVA-specific IgE after CpG treatment in two different strains of mice. Mice were sensitized with OVA intraperitoneally, and immunized with OVA alone, OVA+CpG 5 μg and 50 μg CpG or OVA+ non-CpG. One day before CpG immunization (opened bar) and two weeks after the immunization (closed bar), serum are collected and analyzed by ELISA. Results show the mean±SEM of absorbance at 450 nm from six mice.

/6 following recall stimulation of OVA.

DISCUSSION

According to the recent understanding of the immunopathogenesis of allergic diseases, it

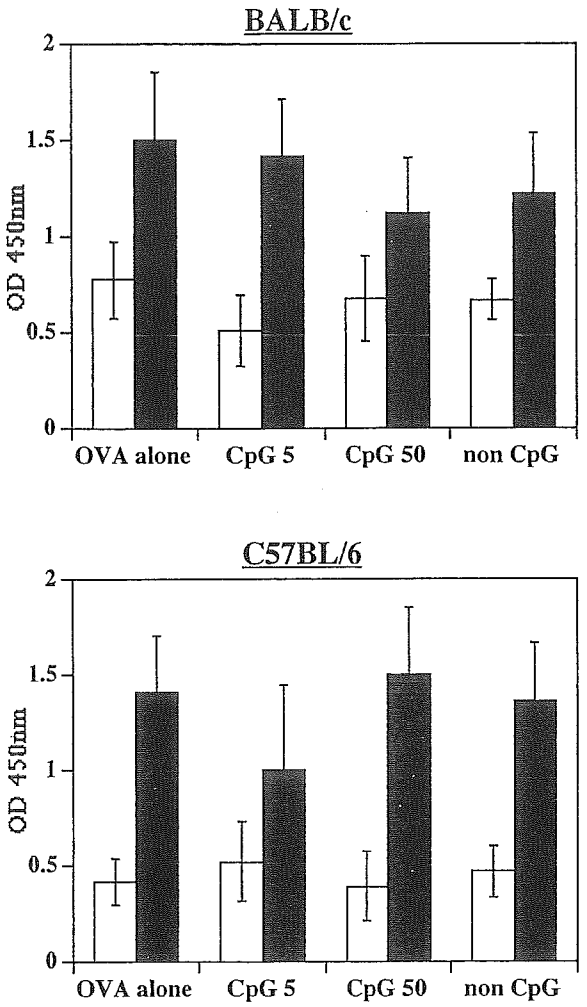

FIG. 3. IgG 1 production after CpG treatment in two different strains of mice. Mice were immunized with OVA alone, OVA+CpG 5 μg and 50 μg CpG or OVA+non-CpG. Mice were immunized with OVA alone, OVA+CpG 5 μg and 50 μg CpG or OVA+non-CpG. Antibodies were measured by indirect ELISA one day before CpG immunization (opened bar) and two weeks after the immunization (closed bar). Results show the mean±SEM of absorbance at 450 nm from six mice.

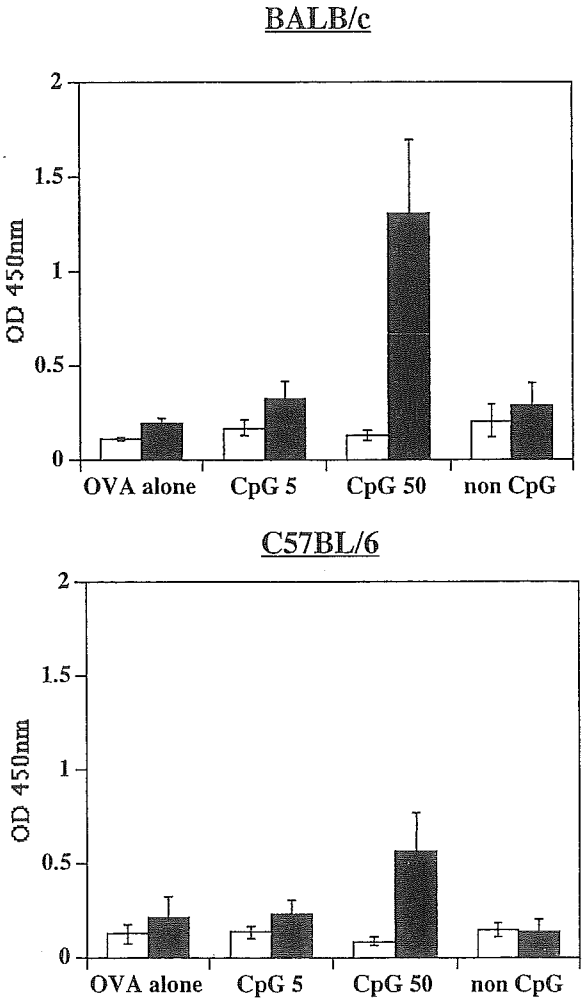

FIG. 4. Serum IgG 2 a following the intranasal immunization with CpG. Mice were immunized with OVA alone, OVA+CpG 5 μg and 50 μg CpG or OVA+non-CpG. Mice were immunized with OVA alone, OVA+CpG 5 μg and 50 μg CpG or OVA+non-CpG. Antibodies were measured by indirect ELISA one day before CpG immunization (opened bar) and two weeks after the immunization (closed bar). Results show the mean±SEM of absorbance at 450 nm from six mice.

is likely that skewing from Th 2 to Th 1 responses is a promising approach in developing new therapeutics. Unmethylated CpG is one of the strong Th 1 adjuvants and could induce mucosa immune response. Takabayashi et al recently demonstrated that intranasal administration of CpG with OVA could prevent airway rather than systemic Th 2 response in mice (25). Hussain et al reported that intraperitoneal immunization with CpG induced suppression of Th 2-