日本熱帯医学会雑誌

第26巻 第4号 平成10年12月

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日熱医会誌

日本熱帯医学会

Jpn. J. Trop. Med. Hyg., Vol. 26, No. 4, 1998, pp. 305-318

HUMORAL IMMUNE RESPONSES AGAINST MEMBERS OF THE HSP70 FAMILY IN *TOXOPLASMA GONDII*

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Received May 11, 1998/Accepted September 21, 1998

Abstract: The cDNA for a member of stress induced 70-kDa protein family (hsp70) from *Toxoplasma gondii* was cloned. The deduced amino acid sequence revealed a 667 amino acid protein 70-80% homologous to other parasite and mammalian hsp70s. Southern blot analysis suggested that it is encoded by an intronless gene. A protein of ~77-kDa was identified in a lysate of *T. gondii* tachyzoites by mAbs generated against the recombinant hsp70 protein. Anti-human hsp70 mAb also cross-reacted with a *T. gondii* protein of the identical molecular weight. However, immunoprecipitation and Western blot analysis of these proteins indicated that it was distinct from the cloned hsp70 product, suggesting that *T. gondii* expresses another hsp70-like protein. Among 22 mice infected with a low virulence Fukaya strain of *T. gondii*, 6 mice exhibited significant humoral anti-hsp70 Ab responses. This Ab responses peaked at 1-2 weeks of infection, plateaued for 2-3 weeks and gradually declined to nearly undetectable levels at 6 weeks of infection. In contrast, the levels of serum Ab specific for soluble tachyzoite Ags continued to increase during the infection in all mice examined. These features suggest that pathogen-derived hsp70 may play a unique role in the induction and maintenance of the host immune responses.
Key words: hsp70, *Toxoplasma gondii*, immune responses, antibody

INTRODUCTION

Members of the stress induced 70-kDa protein family, heat shock protein 70 (hsp70), are expressed in every cell type of eukaryotic as well as prokaryotic cells. They carry out essential functions as molecular chaperones in protein folding, translocation and multimeric polypeptide assembly (reviewed in Gething and Sambrook, 1992; Hartl, 1996; Melnick and Argon, 1995). Each eukaryotic cell expresses several different proteins of the hsp70 family localized in different cellular compartments including the constitutively expressed heat shock cognate protein 70 (hsc70) and stress induced hsp70 in the cytosol, glucose-regulated protein 78 (grp78) in the lumen of the endoplasmic reticulum, and grp75 in the mitochondrial matrix. Consistent with their essential functions, the amino acid sequence of hsp70 is highly homologous among different species. However, hsp70s are immunodominant antigens of many infectious microorganisms. Antibodies against hsp70s have been described in the serum of patients with a variety of pathogens including *Plasmodium falciparum* (Yang *et al.*, 1987), *Leishmania donovani* (MacFarlane *et al.*, 1990), *Trypanosoma cruzi* (Engman *et al.*, 1990), *Schistosoma mansoni* (Hedstrom *et al.*, 1987), and Mycobacterium (Young *et al.*, 1985). T cells from previously infected individuals have been reported to proliferate in response to native *Mycobacterium bovis* hsp70 (Young and Mehara, 1985) as well as recombinant hsp70 (McKenzie *et al.*, 1991). Those studies indicate that hsp70s are potent antigens for both B and T cells during infection with these pathogens. *T. gondii* is an obligate intracellular protozoan parasite which can infect a number of different cell types (Frenkel, 1988; McCabe and Remington, 1988). Infection of healthy individuals with the parasite induces specific antibody production and both class I and class II restricted T cell responses, culminating in powerful protective immunity (Aosai *et al.*, 1994; Yang *et al.*, 1995; Yano *et al.*, 1989). However, the exposed individuals remain chronically infected without apparent symptoms; and the outcome can be fatal if the host's immune system becomes com-

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promised. Considering the importance of the pathogen-derived hsp70 in the induction of immune responses against intracellular organisms, we have cloned a cDNA homologous to hsp70 from a *T. gondii* cDNA library. A panel of mAbs was generated against the recombinant hsp70 protein. The humoral immune response against this hsp70 was investigated in mice infected with a low virulence Fukaya strain of *T. gondii*. The kinetics of the antibody response against hsp70 was distinct from that against other soluble *T. gondii* antigens, suggesting a unique role of pathogen derived hsp70 in triggering and maintaining the host immune response.

MATERIALS AND METHODS

T. gondii and animals:

Tachyzoites of the *T. gondii* RH strain were maintained *in vitro* using the human B cell line ARH. The Fukaya strain was maintained *in vivo* by repeated oral passages of brain cysts containing bradyzoites into B10.A(4R) mice. BALB/c and C57BL/6 (B6) mice were originally purchased from SRC (Shizuoka) and were maintained in the animal facility of Nagasaki University. B10.A(4R) mice have been maintained in our laboratory for sometime.

Isolation of *T. gondii* hsp70 cDNA:

A MOSElox cDNA library was constructed from poly(A)$^+$ RNA isolated from tachyzoites of the *T. gondii* RH strain using a cDNA synthesis module and a cDNA rapid cloning module (Amersham, Buckinghamshire, England) according to the manufacturer's instructions. The library was screened with the radiolabelled human hsp70 DNA probe (ATCC, Rockville, MD) (Hunt and Morimoto, 1985) in 3×SSC, 5×Denhardt's solution, 50 mM Tris Base (pH 7.5), 1 mM EDTA, 0.5% SDS and 20 μg/m*l* denatured salmon sperm DNA at 60°C, and washed in 0.1×SSC and 0.1% SDS at 60°C. From a total of 1×10^5 cDNA clones, 15 clones specifically hybridizing with human hsp70 cDNA were selected. The longest cDNA, pTH14, was subcloned into Bam HI digested pBluescript SKII(+) (Stratagene, La Jolla, CA, U.S.A.), and subjected to DNA sequencing using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.). Analysis of the nucleotide and amino acid sequences was performed using Genetyx-Mac software (Software Development Co., Tokyo).

Southern and Northern blot analysis:

Southern and Northern blottings were performed as described previously (Komori *et al.*, 1993). DNA extracted from tachyzoites of the *T. gondii* RH strain was digested with restriction enzymes, separated on a 1% agarose gel, and transferred to a nylon membrane. A Pst I-Kpn I fragment (nucleotides 430-935) and a PvuII-Hind III fragment (nucleotides 1668-2123) of *T. gondii* hsp70 cDNA were used as probes for the 5′ and 3′ regions, respectively, of the gene. The blot was prehybridized and hybridized with ^{32}P-labeled probe in 5× SSC, 5×Denhardt's solution, 50 mM Tris (pH 7.5), 1% SDS and 50 μg/m*l* salmon sperm DNA for 16 hr at 65°C. The filter was washed twice in 2×SSC/0.1% SDS at 20°C, twice in 0.2×SSC/0.1% SDS at 20°C, and twice in 0.1% SSC/0.1% SDS at 65°C. After being rinsed with 2×SSC, the washed blot was analyzed using a bioimage analyzer, BAS5000Mac (Fujifilm, Tokyo).

RNA was extracted from the RH strain of *T. gondii* by the method described (Chomczynski and Sacchi, 1987), denatured with formaldehyde, subjected to electrophoresis through a 1% agarose gel and transferred to a nylon membrane. The blot was hybridized and analyzed by the method described for Southern blotting.

Expression and purification of the recombinant hsp70 proteins:

The Xho I site was introduced into the 5′ region of the full length *T. gondii* hsp70 cDNA (pTH14) and the ATG start codon was removed by PCR with the oligonucleotide primers TH5′ (5′-catctcgaggcggactctcctgctgt-3′) and T7 using pTH14 subcloned into pBluescript IISK as a template. The PCR product was digested with Xho I and Bam HI and subcloned into the pET15b expression vector (Novagen, Madison, WI, U.S.A.). This plasmid allows the N-terminal fusion of a histidine tag sequence to the *T. gondii* hsp70 protein. N-terminal and C-terminal fragments of hsp70 were also expressed as fusion proteins using pET15b. These constructs were generated by PCR using pTH14 as a template with TH5′ plus 5′-cgggatccttaaccgagagagagagaggcgc-3′ primers, and 5′-catctcgagctggagacagctggtggt - 3′ plus T7 primers, respectively. After digestion with Xho I and Bam HI, each PCR product was subcloned into the pET15b expression vector.

A Bam HI-Hind III digested fragment of the genomic human hsp70 gene (Hunt and Morimoto, 1985) was subcloned into pBluescript SK II. The gene was amplified by PCR using pH1 primer (5′-catctcgaggccaaagccgcggcag-3′) and T7 primer. The PCR product was digested with Xho I (located within the pH1 primer) and Hind III (located within the multiple clon-

ing site of pBluescript SK II) and was subcloned into pGEM7Zf (+) (Promega, Madison, WI, U.S.A.). The DNA fragment containing the hsp70 gene was isolated by digesting this plasmid with Xho I and Bam HI and subcloned into pET15b. The resulting construct can express human hsp70 protein containing the N-terminal fusion of a histidine tag sequence.

Escherichia coli BL21 (DE3) cells harboring the expression plasmid pET15b construct were cultured at 37°C in LB medium supplemented with ampicillin. Expression was induced by the addition of IPTG at a final concentration of 1 mM, and the cells were cultured for an additional 3 hr. The cells were then suspended in 5 mM imidazole, 0.5 M NaCl and 20 mM Tris (pH8.0) and lysed by sonication. The lysate was cleared by centrifugation, and histidine tagged recombinant protein was purified from the supernatant using a ProBond column (Invitrogen, San Diego, CA, U.S.A.) following standard procedures. Briefly, cells were homogenized in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0). After centrifugation, the supernatant was applied to the ProBond column. The column was washed with binding buffer followed by wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 8.0). The bound protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) and was dialyzed extensively against PBS. SDS-PAGE analysis of the purified tagged proteins from full length, N-terminal and C-terminal *T. gondii* hsp70 constructs indicated single major bands of 76-kDa, 46-kDa and 31-kDa, respectively, after Coomassie Blue staining. The yield of the recombinant protein was approximately 1 mg from a 1 liter culture. The protein was concentrated with a Centriprep-30 concentrator (Amicon, Beverly, MA, U.S.A.).

MAbs specific for hsp70:

BALB/c mice were immunized with purified recombinant *T. gondii* hsp70 fusion protein in complete Freund's adjuvant. The booster immunization was performed using the same antigen in incomplete Freund's adjuvant. Spleen cells from the primed mice were fused with SP2/0-Ag14 cells using a cell fusion apparatus type SSH-1 (Shimadzu Co., Kyoto) according to the manufacturer's protocol. Fused cells were selected in medium containing HAT, and the supernatants of the cultures were tested for the presence of anti-hsp70 mAb by ELISA as well as by immunoprecipitation of hsp70 from *T. gondii* lysate. Positive hybridomas were cloned by the limiting dilution method. A mAb specific for human hsp70, 1E11, was generated by the same method except the fusion was performed using polyethylene glycol. The rat anti-hsp70 mAb 7.10 (Kurtz *et al.*, 1986) was purchased from Affinity BioReagents, Inc. (Neshanic Station, NJ, U.S.A.).

ELISA:

The enzyme-linked immunoadsorbent assay was performed as described (Hornbeck, 1996). Briefly, flat bottom microtiter plates (Dynatech, Zug, Switzerland) were coated with recombinant hsp70 protein and soluble tachyzoite Ags in PBS at a concentration of 2 and 40 μg/ml, respectively, at 4°C for more than 24 hr. Plates were blocked with blocking buffer (borate buffered saline containing 0.05% Tween 20, 1 mM EDTA, 0.25% BSA and 0.05% NaN_3), and then incubated with the first antibody. After washing, each well was incubated with alkaline phosphate conjugated anti-mouse Igs (γ and L chain specific) (Tago, Camarillo, CA, U.S.A.) and washed with distilled water before incubation with p-nitrophenyl phosphate substrate solution. Soluble tachyzoite Ags were prepared by extensive dialysis of the lysate of *T. gondii* RH strain tachyzoites with PBS.

Western blotting:

Western blot analysis of hsp70 expression was performed as previously described with a slight modification (Tamura and Yui, 1995). Briefly, protein lysates of *T. gondii* (7.5×10^8 cells/ml) and the human B cell line ARH (2.5×10^7/ml) were prepared in lysis buffer containing 300 mM NaCl, 50 mM Tris•HCl (pH7.5), 0.5% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 1 mM PMSF, and centrifuged at $10{,}000\times g$ for 15 min to remove nuclei. Each sample was separated by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane (NitroBind; MSI, Westboro, MA). Blots were blocked with 10% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBST), probed with anti-hsp70 mAbs in TBST for 1 hr, incubated with biotinylated anti-mouse or anti-rat IgG Ab (Jackson ImmunoResearch, West Grove, PA, U.S.A.) at 1:2,000 for 1 hr, and incubated with horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA, U.S.A.) at 1:3,000 for 30 min. Protein bands were visualized using an ECL detection system (Amersham, Buckinghamshire, England) according to the manufacturer's specifications.

In some experiments, protein purified by immunoprecipitation was used for Western blot analysis. Immunoprecipitation was performed as previously described with some modifications (Yui *et al.*, 1988). Briefly, cells were lysed in lysis buffer containing 0.5%

```
GCGCAAAGGA GTGTAGAAGA TGCATTGTTC GGTCAAGTAT CTGCAAGAAA GAAAGGCTTT CGGAAAGGAA ACCGGGGCTC   80
TGCAAGAAAT TGCCAGTGTG TTTCGTCTTT TTTGGCTTGA ACAGCGAGAT TTGCACCGTG TGCTGCCTCC GCAGCACGGA  160
CAAGTTGCTC CCAGCACCTA CGTCCGAGTA CATACCACAC ACCGCCCTCT TCGTCCCTCA TCATCCCCTG CTGCTGCGCG  240
GGTGTGCTGT CCGGTACGAG TCATAGGGTG TGTCCGGCAC TCGCCCCGCA TTTCATCTTT AGGAGAGTGC CGTTCCGTGC  320
GGTGCAGCTC AAACTAAGGA CGGATCGAAG GTCAGTGTTT TTTCCTTCCA ACCATTTTTC CTTTTCCACT CCCCCGTTCT  400
TTTTTGTGTC GCGTTGCAGT                                                                    420

                                  1                                       10
CGTTTGTCCC TGCAGAAGAC AAC ATG GCG GAC TCT CCT GCT GTG GGT ATT GAC CTT GGC ACC ACC TAT    488
                          MET ala asp ser pro ala val gly ile asp leu gly thr thr tyr

                 20                                      30
TCT TGC GTA GGT GTG TGG AAG AAC GAT GCT GTG GAA ATC ATC GCG AAC GAC CAG GGA AAC AGG ACG  554
ser cys val glu val trp lys asn asp ala val glu ile ile ala asn asp gln gly asn arg thr

         40                                      50
ACC CCG TCC TAC GTC GCG TTC ACC GAC ACG GAG AGA CTT GTC GGT GAT GCT GCG AAG AAC CAA GTC  620
thr pro ser tyr val ala phe thr asp thr glu arg leu val gly asp ala ala lys asn gln val

 60                                      70                                      80
GCA CGC AAC CCG GAA AAC ACC ATT TTC GAT GCC AAG CGC CTA ATC GGT CGC AAG TTT GAT GAT CCC  686
ala arg asn pro glu asn thr ile phe asp ala lys arg leu ile gly arg lys phe asp asp pro

                                     90                                      100
TCG GTC CAG TCG GAC ATG AAG CAT TGG CCA TTC AAG GTC ATT GCT GGT CCG GGA GAC AAG CCC CTC  752
ser val gln ser asp met lys his trp pro phe lys val ile ala gly pro gly asp lys pro leu

                         110                                     120
ATT GAA GTC ACG TAC CAG GGA GAG AAG AAG ACG TTC CAC CCT GAA GAG GTT TGC GCC ATG GTT TTG  818
ile glu val thr tyr gln gly glu lys lys thr phe his pro glu glu val ser ala met val leu

                 130                                     140
GGC AAA ATG AAG GAA ATC GCG GAG GCT TAC CTC GGC AAG GAA GTG AAG GAG GCC GTC ATT ACC GTT  884
gly lys met lys glu ile ala glu ala tyr leu gly lys glu val lys glu ala val ile thr val

         150                                     160
CCT GCG TAC TTC AAC GAT TCG CAG CGT CAG GCT ACC AAG GAT GCT GGT ACC ATT GCC GGC CTC AGC  950
pro ala tyr phe asn asp asr gln arg gln ala thr lys asp ala gly thr ile ala gly leu ser

170                                      180                                     190
GTC CTC CGC ATT ATC AAC GAG CCC ACA GCG GCT GCC ATT GCT TAT GGT CTG GAC AAG AAG GGC TGC 1016
val leu arg ile ile asn glu pro thr ala ala ala ile ala tyr gly leu asp lys lys gly cys

                                     200                                     210
GGT GAG ATG AAC GTC CTC ATC TTC GAC ATG GGT GGC GGT ACG TTC GAT GTG TCG CTG CTT ACA ATC 1082
gly glu met asn val leu ile phe asp met gly gly gly thr phe asp val ser leu leu thr ile
                                                            A
                         220                                     230
GAA GAC GGT ATC TTT GAA GTC AAG GCC ACC GCT GGT GAC ACC CAT CTT GGT GGT GAA GAT TTC GAC 1148
glu asp gly ile phe glu val lys ala thr ala gly asp thr his leu gly gly glu asp phe asp

                 240                                     250
AAC CGT TTG GTG GAC TTC TGC GTC CAG GAC TTC AAG CGC AAG AAC CGC GGA AAG GAC ATC AGC ACC 1214
asn arg leu val asp phe cys val gln asp phe lys arg lys asn arg gly lys asp ile ser thr

         260                                     270
AAC AGC CGT GCC CTT CGT CGC CTG CGT ACC CAG TGC GAG CGC ACC AAG AGA ACT CTC TCT AGC AGC 1280
asn ser arg ala leu arg arg leu arg thr gln cys glu arg thr lys arg thr leu ser ser ser

280                                      290                                     300
ACT CAG GCA ACC ATC GAA ATT GAC TCT CTT TTT GAG GGC ATT GAC TAC TCT GTG TCT ATC TCT CGT 1346
thr gln ala thr ile glu ile asp ser leu phe glu gly ile asp tyr ser val ser ile ser arg
                                                        A
                                     310                                     320
GCG CGC TTT GAG GAG CTT TGC ATG GAC TAC TTC CGC AAC TCC CTG TTG CCC GTC GAG AAG GTC CTC 1412
ala arg phe glu glu leu cys met asp tyr phe arg asn ser leu leu pro val glu lys val leu

                         330                                     340
AAG GAC TCT GGT ATT GAC AAG CGC TCG GTC AGC GAA GTT GTG TTG GTT GGT GGA TCT ACC CGT ATC 1478
```

Figure 1 Nucleotide sequence of the *T. gondii* hsp70 cDNA. Nucleotides are numbered on the right margin and amino acids above the nucleotide sequence. The sequence contains the following underlined elements: (A) potential glycosylation sites; (B) GGMP repeats; (C) AATAAT polyadenylation signal-like sequence. Both strands of the subcloned cDNA in pBluescript SKII(+) were completely sequenced.

```
lys asp ser gly ile asp lys arg ser val ser glu val val leu val gly gly ser thr arg ile

                 350                                     360
CCC AAG ATT CAG CAG CTC ATC ACT GAC TTC TTC AAC GGA AAG GAG CCG TGC AGG TCG ATC AAC CCC  1544
pro lys ile gln gln leu ile thr asp phe phe asn gly lys glu pro cys arg ser ile asn pro

         370                                     380
GAT GAG GCC GTT GCG TAC GGT GCT GCT GTC CAG GCA GCG ATC TTG AAG GGA GTT ACC AGC TCT CAG  1610
asp glu ala val ala tyr gly ala ala val gln ala ala ile leu lys gly val thr ser ser gln

390                                     400                                     410
GTG CAG GAT TTG CTT CTT CTG GAT GTT GCG CCT CTC TCT CTC GGT CTG GAG ACA GCT GGT GGT GTC  1676
val gln asp leu leu leu leu asp val ala pro leu ser leu gly leu glu thr ala gly gly val

                             420                                     430
ATG ACC AAG CTG ATT GAA AGA AAC ACA ACG ATC CCG ACC AAG AAG TCT CAG ACC TTC ACC ACG TAC  1742
met thr lys leu ile glu arg asn thr thr ile pro thr lys lys ser gln thr phe thr thr tyr

                     440                                     450
GCG GAC AAC CAG CCA GGA GTG CTG ATT CAG GTG TAC GAA GGT GAG CGT GCG ATG ACC AAA GAC AAC  1808
ala asp asn gln pro gly val leu ile gln val tyr glu gly glu arg ala met thr lys asp asn

                 460                                     470
AAC CTC CTG GGC AAA TTC CAC CTG GAT GGT ATC CCC CCC GCC CCC CGT GGT GTC CCC CAA ATC GAA  1874
asn leu leu gly lys phe his leu asp gly ile pro pro ala pro arg gly val pro gln ile glu

         480                                     490
GTC ACT TTC GAT ATC GAC GCT AAC GGT ATC ATG AAC GTC ACA GCG CAA GAC AAG TCC ACC GGA AAG  1940
val thr phe asp ile asp ala asn gly ile met asn val thr ala gln asp lys ser thr gly lys
                                                                    A
500                                     510                                     520
AGC AAC CAA ATC ACC ATC ACG AAC GAC AAG GGC CGC CTC AGT GCG TCC GAA ATC GAC CGC ATG GTG  2006
ser asn gln ile thr ile thr asn asp ils gly arg leu ser ala ser glu ile asp arg met val

                             530                                     540
CAA GAG GCA GAG AAG TAC AAA GCC GAA GAC GAA CAG AAC AAG CAC CGT GTG GAG GCG AAG AAT GGC  2072
gln glu ala glu lys tyr lys ala glu asp glu gln asn lys his arg val glu ala lys asn gly

                     550                                     560
CTG GAG AAC TAC TGC TAC CAC ATG AGA CAG ACC TTG GAT GAC GAG AAG CTT AAG GAC AAG ATC TCC  2138
leu glu asn tyr cys tyr his met arg gln thr leu asp asp glu lys leu lys asp lys ile ser

                 570                                     580
TCT GAG GAC AGA GAC ACT GCC AAC AAG GCC ATC CAG GAG GCC CTT GAC TGG CTG GAC AAG AAC CAA  2204
ser glu asp arg asp thr ala asn lys ala ile gln glu ala leu asp trp leu asp lys asn gln

         590                                     600
CTA GCA GAG AAG GAG GAA TTC GAG GCG AAG CAG AAG GAA GTT GAG TCC GTC TGC ACA CCA ATC ATC  2270
leu ala glu lys glu glu phe glu ala lys gln lys glu val glu ser val cys thr pro ile ile

610                                     620                                     630
ACC AAG CTG TAC CAG GCA GGT GCG GCT GCA GGT GGC ATG CCT GGT GGT ATG GGC GGT ATG CCT GGT  2336
thr lys leu tyr gln ala gly ala ala ala gly gly met pro gly gly met gly gly met pro gly

                             640                                     650
GGT ATG GGC GGT ATG CCT GGT GGT ATG GGC GGT ATG CCC GGC GGG ATG GGC GGT ATG CCC GGT GCA  2402
gly met gly gly met pro gly gly met gly gly met pro gly gly met gly gly met pro gly ala
                                                 B
                     660
GGC ATG GGA GGC TCT GGC GGC CCC ACC GTG GAG GAA GTT GAT                                   2444
gly met gly gly ser gly gly pro thr val glu glu val asp

TAACTGTTGA AACGGAAAGA AGTGAACAAA AACCCCATGT GACGTGACAG TTTTTGGGTT CTTCGGAAGA AGAAAGTAAC  2524
CGGAGTTTTG ACTTCTCTCC AGTAGTGGTG TATCGCACAT GCATCAACTT CCGTGGAAGA GAGGTTGAAG AGGGGGAGTC  2604
CTGGTGTATA TACACTGTGT GTATATATAA CATCAACTTC ATATTGTTTT CGAAAGTATC GAAGCATCAA CTTTCTCGCA  2684
AAAAGAAACC CTGCCACCTA TGTGGATGAG TCCCTTGCTG AATAATCCCT AATTC                             2739
                                                   C
```

Figure 1-2

Triton-X and centrifuged to remove nuclei. Each sample was incubated with anti-hsp70 mAb at 4°C for 1 hr, and with protein A Sepharose (Sigma, St. Louis, MO, U.S.A.) for an additional 1 hr, washed, boiled with SDS sample buffer, and separated under reducing conditions by 10% SDS-PAGE. After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane, and the presence of the specific proteins was detected using anti-hsp70 mAbs or mouse serum as described.

The GenBank accession number of the *T. gondii* hsp70 cDNA sequence is U82281.

RESULTS

Structure of *T. gondii* hsp70:

T. gondii hsp70 cDNA was cloned by screening a cDNA library derived from the tachyzoites of the *T. gondii* RH strain with the human hsp70 gene probe (Hunt and Morimoto, 1985). The entire nucleotide sequence (2739 base pairs) of the longest cDNA clone (pTH14) was determined (Fig. 1). The cDNA sequence contained a single large open reading frame predicting a 667 amino acid protein of 72,292 daltons. This coding region was flanked by 443 bp on the 5′ end and by 295 bp

```
T.g. MA---DS----P----AVGIDLGTTYSCVGVWKNDAVEIIANDQGNRTTPSYVAFT-DTERLVGDAAKNQVARNPENTIFDAKRLIGRKFDDPSVQSDMK 93
P.f. ..S.KG.KPNL.ESNI.I..............R.EN.D..................-.....I...............V..........TES.......
T.c. .-----------TYEG.I.............Q.ER...................-.S...I.........M..R..V...........S..V......
M.m. .-----------SKGP...............FQHGK..................-.....I.........M..T..V.........R...AV......
M.l. ..-------------R.........N.V.S.LEGGDPVVV..SE.S.....T...ARNG.V...QP....-.---V-.NV.--.T.-.S-VKRHMG..--

T.g. HWPFKVIAGPGDKPLIEVTYQGEKKTFHPEEVSAMVLGKMKEIAEAYLGKEVKEAVITVPAYFNDSQRQATKDAGTIAGLSVLRIINEPTAAAIAYGLDK 188
P.f. ....T.KS.VDE..M..........L.....I.S...Q....N...F...SI.N.........................N.M...............H.
T.c. .......TKGD...V.Q.QFR..T...N.....S...S.......S....Q..K..V......................E.................
M.m. ....M.V-NDAGR.KVQ.E.K..T.S.Y.....S...T............T.TN..V......................N.................
M.l. -.S--I----------.I--D.--.KYTAQ.I..R..M.L.RD......EDITD....T......A......E..Q....N....V.......L......

T.g. KGCG-EMNVLIFDMGGGTFDVSLLTIEDGIFEVKATAGDTHLGGEDFDNRLVDFCVQDFKRKNRGKDISTNSRALRRLRTQCERTKRTLSSSTQATIEID 286
P.f. ..K.-.K.I....L.................................N...E..........L.K..............A...............
T.c. VED.K.R......L.......T....DG........N................AHFTDE.....K...L...L........A...A......AA.......
M.m. -KV.A.R......L.......I...........S..................M.NHFIAE....HK-....E.K..V.....A...A..........S....
M.l. --GER.QTI.V..L..........E.GE.VV..R..S..N....D.W.D.I.NWL.DK..-GTS.I.LTKDKM.MQ...EAA.KA.IE....QSTSVNLP

T.g. SLFEGID-----YSVSISRARFEELCMDYFRNSLLPVEKVLKDSGIDKRSVSEVVLVGGSTRIPKIQQLITDFFNGKEPCRSINPDEAVAYGAAVQAAIL 382
P.f. .......-----...TV.........I....DT.I........AMM..K..H................T..KE......A.....................
T.c. A..DNV.------FQAT.T........GEL..GT.Q...R..Q.AKM...A.HD............VM..VS...R...LKK..Q.............F..
M.m. ..Y....-----FYT..T.......NA.L..GT.D....A.R.AKL..SQIHDI.............K.LQ.......LNK...................
M.l. YITVDS.KNPLFLDEQLI..E.QRITQ.LLDRTRQ.FQS.V..A..SVSEIDH.........M.AVTD.VKELTG....NKGV....V..V...L..GV.

T.g. KGVTSSQVQDLLLLDVAPLSLGLETAGGVMTKLIERNTTIPTKKSQTFTTYADNQPGVLIQVYEGERAMTKDNNLLGKFHLDGIPPAPRGVPQIEVTFDI 482
P.f. S.DQ.NA.........CS.......................A....I.....................L...................K..........
T.c. T.GK.K.TEG......T..T..I........S..K............I.S.........H...F.........CH...T.E.S....P............L
M.m. S.DK.EN..........T.....I........V..K........QT..L...S..........................E.T..................
M.l. ..---EVKDV...DVTP.....I..K.................R.E....AD....S.Q....Q...EIASH.K...S.E.T..................

T.g. DANGIMNVTAQDKSTGKSNQITITNDKGRLSASEIDRMVQEAEKYKAEDEQNKHRVEAKNGLENYCYHMRQTLDDEKLKDKISSEDRDTANKAIQEALDW 582
P.f. .....L....VE.....Q.H...........QD......ND.........E.RK.I..R.S......GVKSS.E.Q.I.E.LQPAEIE.CM.T.TTI.E.
T.c. .....L..S.EE.G...R...VL........RA..E...R..A..E...KDQVRQID........AFS.KNAVN.PNVAG..EEA.KK.ITS.VE...E.
M.m. .....L..S.V......E.K...........KED.E...............KQRDK.SS..S..S.AFN.KA.VE....QG..ND..KQKILDKCN.IIS.
M.l. .....VH...K..G...E.T.K.QEGS.-..KE......KD..AHAE..RKRREEADVR.QA.TLV.QTEKFVKEQRETENG.RVPE..L..VEAAVAEA

T.g. LDKNQLAEKEEFEAKQKEVESVCTPIITKLYQ--AGAA-AGGMPGGM-GGMPGGM-GGMPG---G--MGGMPGGM-G-GMP-GAGMGGSGGPTVEEVD  667
P.f. .E.....G.D.Y......A....A..MS.I..D-.AG.-........P.......PS....---.--.-NF....P.A...-.NAPA..-........
T.c. .NN..E.S...Y.HR...L.NL....M.NM..GM...GMP.......P.......P.....GMP.GMP.......P.-...G..NPSS.S..E.....
M.m. .....T.......HQ...L.K..N........S-..-GMP......FP..--.APPS.--.ASS.---------------------------..I....
M.l. KTALGGTDISAIKSAMEKLGQDSQALGQAI.EATQA.SKV..-EASAP..-SNSTDDVL-TRRWSTTN.SPK---------------------------
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Figure 2 Alignment of amino acid sequences of *T. gondii* hsp70 (T.g.), *P. falciparum* hsp70 (P.f.) (Yang and Tan-Ariya, 1987), *T. cruzi* hsp70 (T.c.) (Engman and Dragon, 1990), *L. donovani* hsp70 (L.d.) (MacFarlane and Blaxter, 1990), mouse hsc70 (M.m.) (Giebel and Dworniczak, 1988), and *M. leprae* (M.l.) (McKenzie and Adams, 1991). The amino acid sequence of *T. gondii* hsp70 was predicted from the cDNA sequence of clone pTH14 (GenBank accession number U82281). Identical amino acids are indicated by asterisks. Gaps were introduced to maximize homology. Amino acids of *T. gondii* hsp70 are numbered on the right margin.

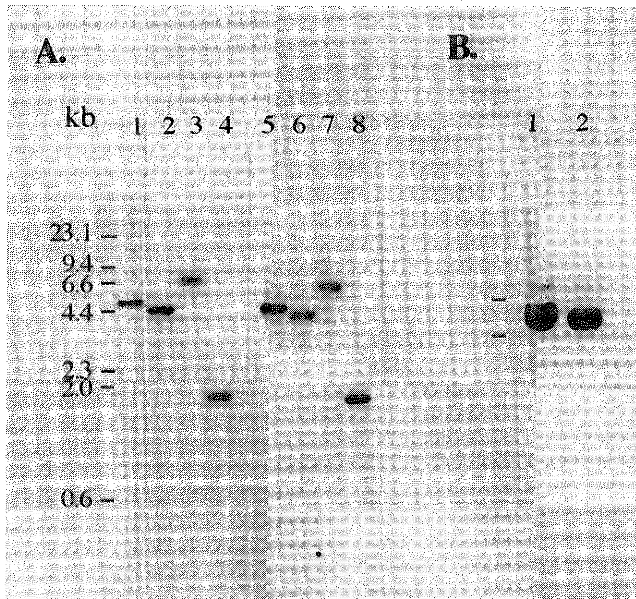

Figure 3 Southern and Northern blot analysis of *T. gondii* hsp70 gene.

A. Analysis of *T. gondii* hsp70 gene by Southern blotting. DNA (3 μg) was cleaved with the restriction enzymes, Bam Hl (1, 5), EcoRl (2, 6), Hind III (3, 7) and Pst I (4, 8). The blot was hybridized with a ^{32}P-labeled Pst I-Kpn I fragment (nucleotides 430-935) (1-4) or a Pvu II-Hind III fragment (nucleotides 1668-2123) (5-8) of pTH14 cDNA. The blot was washed under stringent conditions (0.2×SSC/0.1% SDS at 65°C). Kilobases shown at left.

B. Northern blot analysis of hsp70 gene expression in cultured *T. gondii* tachyzoites. Total tachyzoite RNA (5 μg) extracted from cultured tachyzoites (1) and tachyzoites heat shocked at 42°C for 2 hr (2), was analyzed by blotting. The hsp70 probe was a Pst I-Kpn I fragment spanning the nucleotides 430-935 of pTH14. The positions of rRNA are indicated at left.

on the 3′-untranslated end. The higher eukaryotic polyadenylation signal AATAAA was absent in the 3′ untranslated region of hsp70 cDNA. Instead, the AATAAT sequence was found 10 nucleotides upstream of the poly (A) tail. The deduced primary sequence was 80.8%, 72.3%, 72.3% and 74.1% homologous to *P. falciparum* hsp70 (Yang and Tan-Ariya, 1987), *T. cruzi* hsp70, *L. donovani* hsp70 (MacFarlane and Blaxter 1990), and human and rodent heat shock cognate proteins (hsc70) (Dworniczak and Mirault 1987; Giebel *et al.*, 1988), respectively, indicating that this cDNA encodes *T. gondii* hsp70. The homology to *M. leprae* was 46.1%. Inspection of the amino acid sequence revealed the absence of long hydrophobic stretches and the N-terminal signal sequence. There were three potential N-linked glycosylation sites in the deduced amino acid sequence. At the C-terminal end, there were four complete and one incomplete GGMPGGM repeated sequences. Similar GGMP repeats are present in the majority of parasite hsp70 proteins as well as human and rodent hsc70 (Fig. 2). *T. cruzi* and *P. falciparum* hsp70 contain 9 and 5 GGMP repeats, respectively. Human and mouse hsc70 contain 2 repeats. The C-terminal regulatory motif, EEVD, was conserved as in hsp70s of other eukaryotic cells (Freeman *et al.*, 1995).

The *T. gondii* gene encoding hsp70 was analyzed by Southern blotting (Fig. 3A). The DNA was digested with restriction enzymes and probed with two cDNA probes; one spanning nucleotides 430-935 (corresponding to amino acids Met-1 to Thr-164) and the other 1668-2123 (Thr-407 to Leu-560). These probes hybridized with the identical restriction fragments of the hsp70 gene. Only single bands of approximately 4.9, 4.5, 7.0 and 1.9 kb were detected in Bam Hl, Eco Rl, Hind III, and Pst I fragments of the DNA after high stringency washing (0.1×SSC, 0.1% SDS at 65°C). The size of the Pst I fragment was identical to the distance between the two Pst I sites of the cDNA sequence (nucleotide 431-2300), implying that there is no intron between these two sites. The blot was also washed at low stringency (0.2×SSC, 0.1% SDS at 20°C) after hybridization, but no additional signals were detected. The expression of this

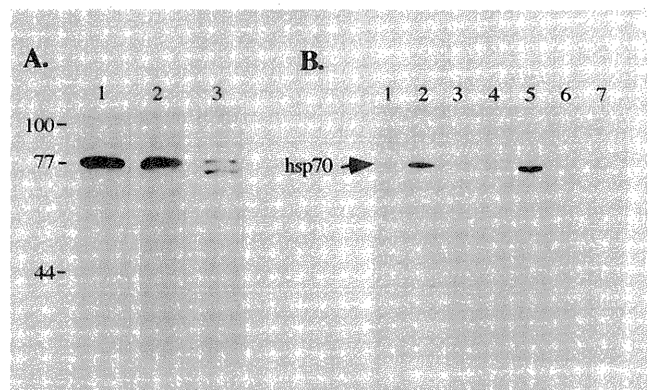

Figure 4 Identification of the native *T. gondii* hsp70 by mAbs.

A. Lysate of *T. gondii* RH strain tachyzoites was separated by 10% SDS-PAGE, blotted, and probed with mAbs T×D11 (1), T×A5 (2) and 7.10 (3). Molecular weights are indicated on the left.

B. Lysate of *T. gondii* tachyzoites was immunoprecipitated with no Ab (1), T×D11 (2), T×G3 (3), T×B12 (4), T×H2 (5), T×A5 (6) and 1E11 (7); separated by 10% SDS-PAGE; blotted; and probed with anti-hsp70 mAb 7.10.

Table 1 ELISA of anti-hsp70 mAbs produced in this study

Immunization**	Name	Isotype	Binding of mAb with recombinant proteins prepared as*			
			T. gondii hsp70	N-terminal fragment	C-terminal fragment	human hsp70
hsp70	T×D11	γ2b	+	−	+	−
	T×G3	γ1	+	−	+	−
	T×B12	γ1	+	−	+	−
	T×H2	γ2a	+	−	+	−
	T×A5	γ1	+	+	−	+
human hsp70	1E11	γ2b	−	N.D.***	N.D.	+

*The specificity of each mAb was tested by ELISA using total *T. gondii* hsp70, N- as well as C-terminal fragments of the hsp70, and human hsp70 recombinant proteins.
The results were measured using plate reader with a 405 nm filter.
+indicates positive results. −indicates the value is within the mean value of the control (without Ab) ±standard error of the mean.
**BALB/c mice were immunized with recombinant *T. gondii* hsp70 or human hsp70 protein. After fusion of spleen cells with SP2, hybridoma supernatants were screened by ELISA using the same antigen.
***not done

gene was determined by Northern blot analysis (Fig. 3B). There was no significant increase in the expression level of hsp70 RNA after treatment of the tachyzoites at 42°C for 2 hr.

Identification of *T. gondii* hsp70 by mAbs:

cDNA (pTH14) was expressed in *E. coli* as a tagged protein carrying six histidine residues at its N-terminal, and was purified by immobilized nickel affinity chromatography. Mice were immunized with this recombinant protein and spleen cells were fused with SP2 cells to produce hybridomas. The initial screening of the hybridoma supernatant was performed by ELISA using the recombinant protein. The hybridomas secreting specific antibodies were further screened for their ability to bind to natural *T. gondii* protein. *T. gondii* lysate was immunoprecipitated with each hybridoma supernatant, separated on SDS-PAGE, blotted, and probed with antisera of the immunized mice. Five hybridomas producing mAb that can bind recombinant as well as natural *T. gondii* protein were cloned by limiting dilution (Table 1). Western blot analysis was performed to determine the *T. gondii* protein detected by these mAbs (Fig. 4). T×D11 and T×A5 bound only an ~77-kDa protein in the *T. gondii* lysate. Anti-hsp70 mAb 7.10 which is widely cross-reactive with members of the hsp70 family including hsp70, hsc72, grp78 and heat inducible hsp72 (Kurtz and Rossi, 1986) detected the ~77- and ~72-kDa proteins expressed in *T. gondii* tachyzoites. To determine whether ~77-kDa *T. gondii* protein identified by our mAbs is identical to that detected by anti-hsp70 mAb 7.10, *T. gondii* lysate was immunoprecipitated with the panel of mAbs, separated on SDS-PAGE, blotted, and was probed with 7.10. All of them specifically precipitated the ~77-kDa protein from *T. gondii* lysate that can bind to 7.10, although the intensities of the bands were not equivalent (Fig. 4B). Since this was the only band that could be detected by T×D11 and T×A5, we concluded that these mAbs specifically bind to the natural hsp70 expressed in *T. gondii*. The expression of this protein was not signifi-

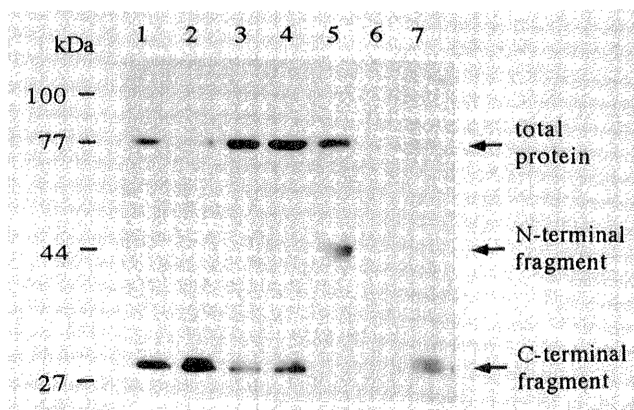

Figure 5 Western blot analysis of the specificity of anti-hsp70 mAbs to recombinant hsp70 fragments. The mixture of purified recombinant hsp70, N- and C-terminal fragments was separated by 10% SDS-PAGE. After blotting, lanes were cut and each strip was probed with mAb T×D11 (1), T×G3 (2), T×B12 (3), T×H2 (4), T×A5 (5), 1E11 (6) and 7.10 (7). Molecular weights are indicated on the left.

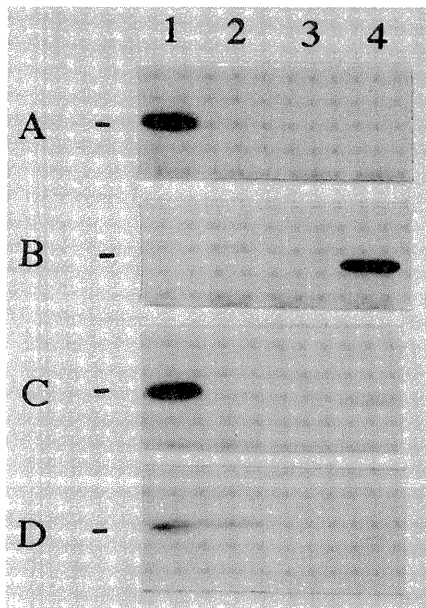

Figure 6 Immunoprecipitation of *T. gondii* hsp70s by T×D11 and by 1E11. Cell lysates of *T. gondii* tachyzoites (1, 2) and the human B cell line ARH (3, 4) were immunoprecipitated with T×D11 (1, 3) or 1E11 (2, 4). Each precipitated sample was split into 4 groups and separated by 10% SDS-PAGE generating 4 identical gels (A-D). Each gel was blotted onto a nitrocellulose membrane and probed with T×D11 (A), 1E11 (B), T×A5 (C) and 7.10 (D). The position of the 77-kDa molecular size marker is indicated as (−).

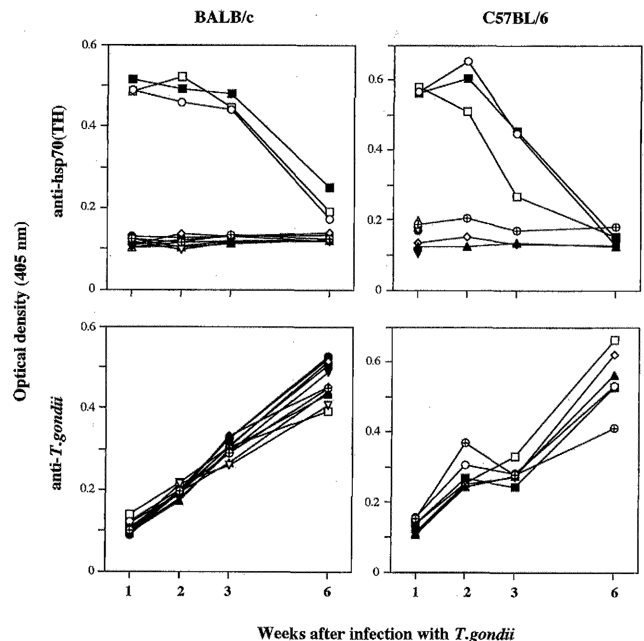

Figure 7 ELISA of anti-hsp70 Ab in the serum of mice infected with *T. gondii*. Serum samples were obtained from each of 11 BALB/c and C57BL/6 mice 1, 2, 3, and 6 weeks after oral infection with 20 cysts of *T. gondii* Fukaya strain as indicated. The levels of antibody against hsp70 and soluble tachyzoite antigens were determined by ELISA as described in Materials and Methods. Serum samples were used at 1:50 dilution. The means of triplicate or duplicate experiments are shown. Five C57BL/6 mice died between 1 and 2 weeks after infection.

cantly altered after treatment of *T. gondii* for 2, 4, or 6 hrs at 42°C (data not shown). MAb1E11, which was originally generated using recombinant human hsp70, also precipitated the protein of the same molecular weight that can bind to 7.10 (Fig. 4B). This protein, however, was not identical to hsp70 detected by *T. gondii* hsp70 specific mAb T×D11, as will be discussed later.

Proteins of the hsp70 family consist of two domain structures: an N-terminal ATPase and a C-terminal peptide-binding domain (Chappell *et al.*, 1987). To determine the domain to which these mAbs bind, we created recombinant N-terminal and C-terminal hsp70 peptides both tagged with six histidine residues. The N-terminal fragment spanned from amino acid Ala-2 to Gly-404, and the C-terminal fragment from Leu-405 to Asp-667. These peptides were affinity purified using nickel chromatography and were used for ELISA and Western blot assays (Table 1, Fig. 5). Of 5 mAbs specific for the hsp70, 4 mAbs bound to the C-terminal peptide binding domain and 1 mAb, T×A5, bound to the N-terminal ATPase domain. Interestingly, T×A5 mAb cross-reacted with recombinant human hsp70, while none of the other 4 mAbs bound to it (Table 1). Anti-human hsp70 mAb, 1E11, did not cross-react with the recombinant *T. gondii* hsp70 protein. The 7.10 mAb bound to the C-terminal fragment of the recombinant hsp70 protein (Fig. 5).

Distinction of *T. gondii* hsp70s precipitated by T×D11 and 1E11 mAbs:

Although Western blot analysis of *T. gondii* hsp70 indicated that 1E11 can bind to the hsp70-like molecule expressed in *T. gondii* (Fig. 4), 1E11 did not bind to the recombinant hsp70 (Fig. 5). To determine that the molecule detected by 1E11 mAbs is distinct from the cloned hsp70 gene product, each protein was purified by immunoprecipitation with 1E11 and T×D11 mAb. The precipitated materials were split into four groups, separated by SDS-PAGE, blotted, and probed with four distinct anti-hsp70 mAbs, T×D11, 1E11, T×A5 and 7.10 (Fig. 6). Human hsp70 was used as a control for the

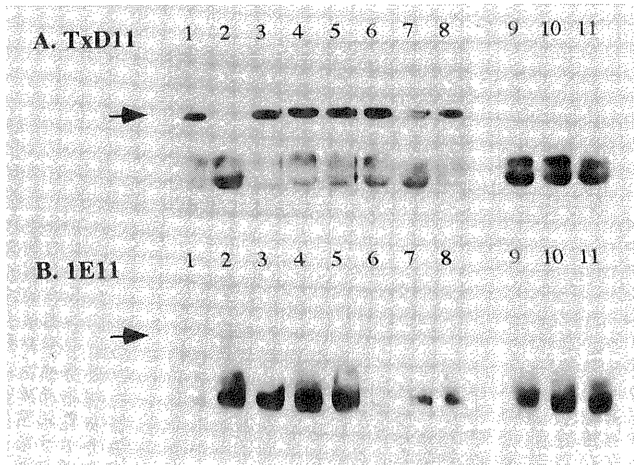

Figure 8 Western blot analysis of anti-hsp70 Ab in the serum of mice infected with *T. gondii*. Lysate of *T. gondii* tachyzoites was immunoprecipitated with T×D11 (A.) or 1E11 (B.) and separated on 10% SDS-PAGE. After blotting, lanes were separated and each strip was probed with mAb T×D11 (1), 1E11 (2), sera from individual BALB/c (3-5) or C57BL/6 (6-8) mice infected for 1 week, and BALB/c mice (9-10) infected for 6 weeks. Serum samples from mice which produced detectable levels of anti-hsp70 Ab by ELISA were used in this experiment.

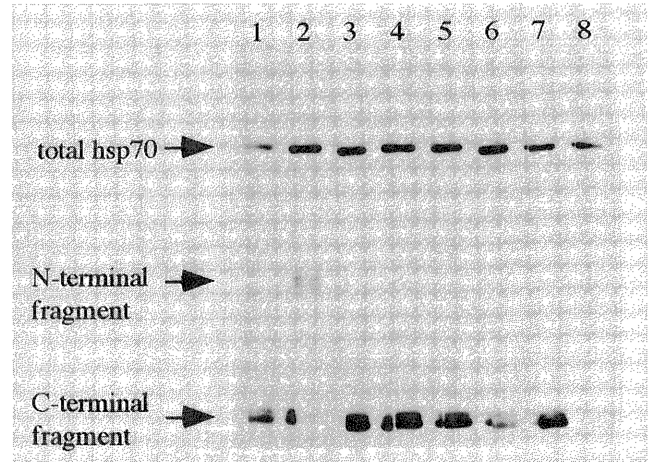

Figure 9 Western blot analysis of the specificity of anti-hsp70 serum from infected mice. The experiment was performed as described in the legend of Fig. 5. The blot of total *T. gondii* recombinant hsp70 as well as N-terminal and C-terminal fragments of hsp70 was cut, and each strip was probed with mAb T×D11 (1), T×A5 (2), serum from individual BALB/c (3-5) or B6 (6-8) mouse infected for 1 week. The serum samples used here were identical to those used in Fig. 8.

immunoprecipitation. The protein precipitated by T×D11 was detected by T×A5 and 7.10 mAb, but not by 1E11. 1E11 precipitated a *T. gondii* protein as detected by itself (B-2) and 7.10 mAb (D-2), although the intensity of the band was weak. This molecule is likely another member of the hsp70 family, because it can bind to anti-hsp70 mAbs 1E11 and 7.10, and because it has a molecular weight similar to the cloned hsp70. This hsp70-like molecule is not humam hsp70 that could have contaminated during the propagation of *T. gondii in vitro,* because the molecular weight of the *T. gondii* protein precipitated by 1E11 was larger than that of the human hsp70 (B-4). MAbs T×D11 and T×A5 did not bind to this protein (A-2, C-2). These reslts suggest that *T. gondii* tachyzoites express another hsp70-like protein in addition to the cloned hsp70. These two molecules can be specifically identified by T×D11 and 1E11 mAb.

Detection of anti-hsp70 Ab in the serum of *T. gondii* infected mice:

Pathogen-derived hsp70s have been shown to be strongly immunogenic to the host immune system in several infectious diseases. To determine whether *T. gondii* hsp70 can be a target of the host immune responses, we examined whether anti-hsp70 Ab can be detected in the serum of mice infected with *T. gondii.* BALB/c and C57BL/6 mice were infected with the low virulence Fukaya strain of *T. gondii*, and serum samples were collected 1, 2, 3, and 6 weeks after infection. ELISA of these samples was performed using plates coated with the recombinant hsp70 (Fig. 7). Antibody to soluble tachyzoite antigens was also examined as a control. In both BALB/c and C57BL/6 mice, significant levels of anti-hsp70 antibody were detected in the sera of 3 out of 11 mice infected. The antibody levels were maintained for 2-3 weeks and gradually declined thereafter. Six weeks after infection, the serum Ab levels became nearly undetectable in C57BL/6 mice. In contrast, the levels of serum antibody specific for soluble tachyzoite antigens continued to increase during the infection in all mice we have examined. During the course of the study, 5 C57BL/6 mice died between 1 and 2 weeks of infection. Interestingly, all 3 mice which developed anti-hsp70 antibody survived while only 3 out of 8 mice which did not developed anti-hsp70 antibody did. Therefore, to determine whether there is any correlation between the ability to produce anti-hsp70 Ab and protection against *T. gondii* infection, we sacrificed these C57BL/6 mice 7 weeks after infection and determined the number of brain cysts. There was, however, no significant difference in the cyst number between the

mice which developed an anti-hsp70 antibody response (803±153 cysts/brain) and those which did not (790±361 cysts/brain).

Characterization of the anti-hsp70 antibody in *T. gondii*-infected mouse serum:

To determine that the anti-hsp70 antibody detected in the serum from infected mice by ELISA did indeed bind to the natural *T. gondii* protein, we purified native hsp70 by immunoprecipitation with T×D11, separated it on SDS-PAGE, blotted and probed with the serum from the infected mice (Fig. 8A). As expected, the antisera obtained from mice one week after infection bound to the natural *T. gondii* hsp70. The antisera from the same mice 6 weeks after infection barely bound to this protein. In a parallel study, we also determined whether these sera contained antibody specific for the hsp70-like protein purified by 1E11 mAb. The protein isolated from *T. gondii* by immunoprecipitation with 1E11 mAb was probed with the same sera as used for the detection of hsp70 (Fig. 8B). These sera did not show any significant binding to hsp70-like protein purified by 1E11 mAb. Finally, to determine whether anti-hsp70 antibody in the sera of the infected mice bound to the ATPase or peptide binding domain of the protein, recombinant N-terminal and C-terminal fragments of hsp70 were used for the Western blot assay (Fig. 9). All 6 serum samples from both BALB/c and C57BL/6 mice bound to the C-terminal but not to the N-terminal fragment of hsp70, indicating that the major antigenic determinant exists in the C-terminal portion of hsp70.

Discussion

Full length cDNA encoding *T. gondii* hsp70 was cloned by screening the *T. gondii* cDNA library with the human hsp70 probe. Southern blot analysis suggested that the gene is located at a single locus in the *T. gondii* genome. This gene appears to lack an intron between nucleotides 431-2300, which covers 92.8% of the coding sequence, suggesting that the *T. gondii* hsp70 gene may be encoded by one continuous open reading frame, similar to the human hsp70 gene (Hunt and Morimoto, 1985). In the 3′ untranslated region, the canonical poly (A) signal AATAAA is not present, although a similar AATAAT sequence is present 10 nucleotides upstream of the poly (A). This signal is also absent in transcripts of other *T. gondii* genes including that of the major surface antigen P30 (Burg *et al.*, 1988).

The overall homology of the deduced amino acid sequence was highest with *P. falciparum,* consistent with their close evolutionary relationship (Kohler *et al.*, 1997). This *T. gondii* hsp70 may be a cytosolic protein because it lacks a signal sequence and ER-retention signal. There were three possible N-glycosylation sites. It is unclear whether these sites are glycosylated, but comparison of the molecular size of the native and recombinant hsp70s (Figs. 4, 5) suggests that there is no extensive glycosylation in these sites. An interesting feature of the deduced amino acid sequence was the presence of multiple repeated sequences; four complete and one incomplete GGMPGGM sequence near the C-terminus. Similar GGMP repeated sequences were observed in all the members of the parasite cytosolic hsp70s that have been cloned to date as well as human and mouse hsc70s. The number of repeats in *T. gondii* hsp70 is particularly high among these proteins and is at a similar level to other protozoan parasites such as *P. falciparum* and *T. cruzi.* The function of this repeated sequence is unknown but may have a role in adaptation of the parasites to the changing environment during their developmental stages within hosts and vectors.

Western blot analysis of the *T. gondii* lysate with anti-hsp70 mAb, T×D11, indicated that *T. gondii* tachyzoites cultured *in vitro* express this protein. The anti-human hsp70 mAb 1E11 also identified a protein of the same molecular weight. The reciprocal binding of these two mAbs, however, indicated that they are distinct proteins. Although we were unable to detect multiple signals in Southern blot analysis of the *T. gondii* genome with the hsp70 probe under a low stringency condition, the protein identified by 1E11 is likely a member of the hsp70 family because it can specifically bind to two anti-hsp70 mAbs (1E11 and 7.10) and because of its molecular size. The nature of this protein is unclear and its further understanding may require molecular cloning of the corresponding gene. Lyons and Johnson identified 65- and 70-kDa proteins expressed in *T. gondii* using polyclonal antiserum against the hsp70 of *P. falciparum* (Lyons and Johnson 1995). The hsp70s identified by our mAbs are clearly distinct from their 70-kDa protein which was expressed in tachyzoites only *in vivo* and not *in vitro.* Their 65-kDa protein was similar to the hsp70s identified here in that it is constitutively expressed, although their molecular weights are clearly different. The side by side comparison of the molecular size or the sequential immunoprecipitation with the two Abs will determine whether they are indeed different.

In an attempt to understand the role of *T. gondii*-derived hsp70 in stimulating host immune responses, we investigated the ability of mice infected with *T. gondii*

to generate humoral immune responses against pathogen-derived hsp70. Two distinct features were observed when compared to the response against soluble tachyzoite antigens. First, the kinetics of the antibody response was quite different. Serum antibody against hsp70 reached peak levels in the acute phase of the infection (1-2 weeks after infection) and then gradually declined. In the chronic phase, the antibody declined to levels barely or not detectable by ELISA or Western blotting. In contrast, the levels of serum antibody against soluble tachyzoite antigen continued to rise during the acute and chronic phases of infection. Several possibilities may account for these kinetic differences. Antibodies to pathogen-derived hsp70 is often cross-reactive to hsp70 of other species including self proteins. The early anti-hsp70 response could be due to the cross-priming of the reactive lymphocytes by hsp70 of other species prior to infection by *T. gondii*. The early clearance of the Ab may also be explained by the absorption of crossreactive Abs by host-derived hsp70. Alternatively, the kinetic differences might be due to the molecular mechanisms by which hsp70 may be presented to lymphocytes in a manner distinct from other soluble tachyzoite antigens. Proteins of the hsp70 family appear to have unique roles in the induction of immune responses such as carrier effects for its associated molecules (Barrios *et al.*, 1992; Udono and Srivastava, 1993). Finally, *T. gondii* become encysted bradyzoites with their growth limited in the chronic phase of infection. Our preliminary study suggests that the bradyzoites express hsp70. However, this antigen may become inaccessible to immune recognition leading to failure to maintain the antibody level.

The second difference of the antibody response to *T. gondii* hsp70 was that only 3 out of a total of 11 infected mice showed significant anti-hsp70 antibody responses in both BALB/c and C57BL/6 mice, while all mice exhibited similar responses against soluble tachyzoite antigens. Age and sex matched mice were used in the experiments, so that it is unlikely to explain these differences. Mice were housed in the same environment and were infected with the same group of cysts. We currently do not know why only some of the infected mice generated significant anti-hsp70 antibody responses. During the course of this study, 5 C57BL/6 mice died between 1 and 2 weeks of infection. Interestingly, all 3 mice that exhibited anti-hsp70 antibody responses survived. However, we did not find any differences in the number of brain cysts between the mice which produced anti-hsp70 antibody during the acute phase of the infection and those that did not. More detailed study will be required to determine whether there is any correlation between the ability of mice to produce anti-hsp70 antibody and protection against *T. gondii* infection.

The majority of the antibodies produced during acute infection with *T. gondii* appear specific for the C-terminal domain of hsp70, suggesting that the C-terminal peptide binding domain contains the dominant B-cell epitope. In agreement with this possibility, among our panel of 5 mAbs specific for *T. gondii* hsp70, 4 were specific for the C-terminal and only one was specific for the N-terminal portion of hsp70. Furthermore, antibody responses against *M. leprae* hsp70 were previously shown to be directed mainly to the C-terminal sequences (Davenport *et al.*, 1992). The majority of the antibodies specific for pathogen-derived hsp70 appear directed towards non-conserved sequences of the protein (Engman and Dragon, 1990). However, differences in overall homology in the C- and N-terminal sequences may not be sufficient to explain the immunodominancy of the C-terminal portion. The homology of the amino acid sequence between mouse and *T. gondii* hsp70 is 76.7% in the N-terminus (Ala-2 to Gly-404) and 70.5% in the C-terminus (Leu-405 to Asp-667). When the GGMP repeated region is excluded, the homology in the C-terminal sequence (Leu-405 to Gln-614) is 73.3%. It would be crucial to determine the dominant epitope to understand the molecular basis underlying the immunodominancy of this protein.

Finally, it has been proposed that mammalian hsp70 participates in the processing of peptide antigens within antigen presenting cells (DeNagel and Pierce, 1992; Srivastava, 1993). The members of the hsp70 family of infectious organisms have a conserved structure. They may not only become targets of the immune response but also modulate the presentation of pathogen-derived antigens to specific T cells. With molecular probes now being available, it would be of interest to learn the molecular and cellular mechanisms underlying the stimulation and modulation of the host immune response by a member of the pathogen-derived hsp70s.

ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan.

We greatly appreciate for the critical comments and discussions from Drs. K. Yamashita and F. Aosai. We also thank Mr. M. Ueda for his excellent technical help, and Dr. G. Massey for correcting English.

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BACTERIOLOGICAL STUDY ON *VIBRIO PARAHAEMOLYTICUS* ISOLATED FROM THE OUTBREAKS OF DIARRHEA IN LAOS, AN INLAND COUNTRY

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Received August 24, 1998/Accepted October 21, 1998

Abstract: An epidemic of *Vibrio parahaemolyticus* gastroenteritis with several outbreaks occurred in Vientiane, People's Democratic Republic of Lao (Laos), an inland country in August and September in 1997. Serotypes of the 36 isolated *V. parahaemolyticus* strains from the patients were all O3:K6. The organisms grew in peptone water supplemented with 10% NaCl. All isolates were positive for Kanagawa phenomenon, and they were positive for *tdh,* negative for *trh* and urease. Genomic patterns were not completely identical, as determined by pulsed-field gel electrophoresis. The causative food was suspected to be a papaya salad containing minced and salted fresh water crab. Although a Kanagawa phenomenon positive *V. parahaemolyticus* strain was isolated from the suspicious food, the serotype was O2:K28, *tdh* and *trh* were negative.

INTRODUCTION

Vibrio parahaemolyticus is a halophilic gram-negative rod, and is commonly present in coastal waters throughout the world if the water is not too cold. It is an important causative agent of food-born infection characterized by severe cramping, abdominal pain, and explosive watery diarrhea. The illness is closely associated with the ingestion of seafood, and the illness has rarely been identified in area distant from the coast where sea food is not readily available. However, as proceeding the borderless era, seafood has become available to people in areas far from the sea where this illness had been formerly uncommon. People's Democratic Republic of Lao (Lao PDR, or Laos) is an inland country sharing borders with 5 countries. In August and September, 1997, a few outbreaks of diarrhea due to *V. parahaemolyticus* occurred in Vientiane city. *V. parahaemolyticus* was isolated from 36 diarrhea specimens out of 53 examined. Some patients had ingested salted minced fresh-water crab with papaya salad on the day before developing the illness. *V. parahaemolyticus* was isolated from the crab which was a domestic product from a nearby fresh water river. Considering that *V. parahaemolyticus* O3: K6 may have become prevalent in Southeast Asia recently (Okuda *et al.,* 1997), it is no wonder the infection occurred in Lao PDR. In this communication, the details of the isolates in Lao PDR are described.

MATERIALS AND METHOD

Patients and bacterial strains: Several outbreaks of diarrhea occurred in Vientiane, Lao PDR, in August and September, 1997. The total number of patients is unclear, but the patients were treated in four hospitals. One hospital (Chanthabory) accepted 63 patients, 31 of them were examined for stool culture and 22 were positive for *V. parahaemolyticus*. Another hospital (Sikhotabong) accepted 41 patients, 8 of them were examined and 4 were positive. The number of patients at the other 2 hospitals, Sethatirath and Mahosot, were not reported, but 8 of 8 and 2 of 6 stool samples from these hospitals were positive, respectively. Among a total of 53 stools examined, 36 were positive for *V. parahaemolyticus*. Age distribution of the patients with

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positive *V. parahaemolyticus* was 5 to 70 years old with an average of 25.6 years old. The male to female ratio was 19 to 17. Other than these 36 isolates of *V. parahaemolyticus*, one isolate from the suspected food which was submitted by a patient at Sikhotabong Hospital, and one stocked strain isolated from a patient in Laos in 1993 were also examined.

Biological characterization: Biological behaviors of the organisms to determine the species were examined by routine laboratory techniques. Additionally, productivity of urease was examined using urea medium (Eiken). Hemolytic activity was examined using Wagatsuma medium (peptone 10 g, yeast extract 5 g, mannitol 5 g, K_2HPO_4 5 g, NaCl 70 g, crystal violet 1 μg, agar 15 g, Aq. dist. 1,000 ml, pH 7.5) with human and sheep erythrocytes.

Serotype: Commercialized diagnostic sera (Denka Seiken Co.) were used to determine O and K serogroup. The organisms cultured overnight on nutrient agar supplemented with 3% NaCl were examined by the slide agglutination method.

Gene detection: The presence or absence of the *tdh* and *trh* genes of the isolates were determined by polymerase chain reaction (PCR). The primers for *tdh*, VP21 (5'-TGGTTGACATCCTAGATGACTGTG-3') and VP22 (5'-GGGGATCCCTCAGTACAAAGCCTT-3') were used for amplifying 396 bp of *tdh* with the PCR condition described by Lee and Pan (Lee and Pan, 1993). Primers for *trh*, trh-s (5'-GGCTCAAAATGGTTAAG-CG-3') and trh-r (5'-CATTTCCGCTCTCATATGC-3') were used for amplifying 250 bp of *trh* with the PCR conditions described by Tada *et al.* (1992).

Analysis of chromosome: The chromosomal type of the strains were examined by pulsed-field gel electrophoresis (PFGE) as described by Wong *et al.* (1996). Briefly, whole cells were embedded in 1.5% low-melting point agarose gel blocks for PFGE. The blocks were treated with lysis solution containing lysozyme (1 mg/ml) and N-sodium lauryl sarcosine (1 mg/ml) at 37°C for 48 hr. Then, the blocks were treated with proteinase K solution containing 0.5 mg/ml of proteinase K, 0.5 M EDTA and 10 mg/ml of sodium lauryl sarcosine at 45°C for 48 hr. The blocks were washed 3 times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Finally the blocks were immersed in a solution containing 10 U of *Sfi*I restriction enzyme at 4°C for 16 hr and then 37°C for 48 hr. Electrophoresis was carried out using a CHEF-DRIII system (Bio-Rad) in gels of 1% agarose for 20 hr at 6 V/cm at 14°C with a ramp time of 3 to 80 sec.

Drug susceptibilities: All isolates were examined for susceptibility to ampicillin (ABPC, Meiji Seika), tetracycline (TC, Wako Pure Chemicals), ofloxacin (OFLX, Daiichi Pharmaceuticals), erythromycin (EM, Dainihon Pharmaceuticals), and cefdinir (CFDN, Fujisawa Pharmaceuticals). Minimum inhibitory concentrations (MICs) of the drugs were examined by a plate dilution technique. The plates of heart infusion agar supplemented with 3% NaCl and 1 μg/l of crystal violet, containing the drug at the serial 2-fold concentration from 0.2 μg/ml to 100 μg/ml were prepared. The organisms in HIB supplemented with 3% NaCl cultured overnight were diluted 1:10 with normal saline solution for the inoculum. The organisms were inoculated using a micro planter (Sakuma Co. MIT#00257), and the determination of MIC was made after incubation at 37°C for 24 hr.

RESULTS

Phenotypes of the isolates: All isolates from the patients revealed the same phenotype, showing the typical behaviors of *V. parahaemolyticus*, except for weak growth in 10% NaCl-peptone water. Swarming was

Table 1 Characterization of the isolates

	Vibrio parahaemolyticus strains from		
	outbreaks	suspicious food	patient in 1993
Growth on TCBS	+	+	+
Acid from			
glucose	+	+	+
sucrose	–	–	–
lactose	–	–	–
Decarboxylation			
lysine	+	+	+
ornithine	+	+	+
arginine	–	–	–
Production of			
cytochrome oxidase	+	+	+
Urease	–	–	–
Indol	+	+	+
H_2S	–	–	–
VP	+	+	+
Utilize citrate	+	+	+
Motility	+	+	+
Growth in NaCl			
0%	–	–	–
6%	+	+	+
10%	(+)*	–	–
Kanagawa phenomenon	+	(+)**	+
Serotype	O3:K6	O2:K28	O4:K63

* All 36 isolates from the outbreaks grew in peptone water supplemented with 10% NaCl. That growth ability was maintained for about 6 months after isolation, and then it disappeared.

** Hemolysis on Wagatsuma medium by the strain isolated from the suspected food was weaker than the other strains.

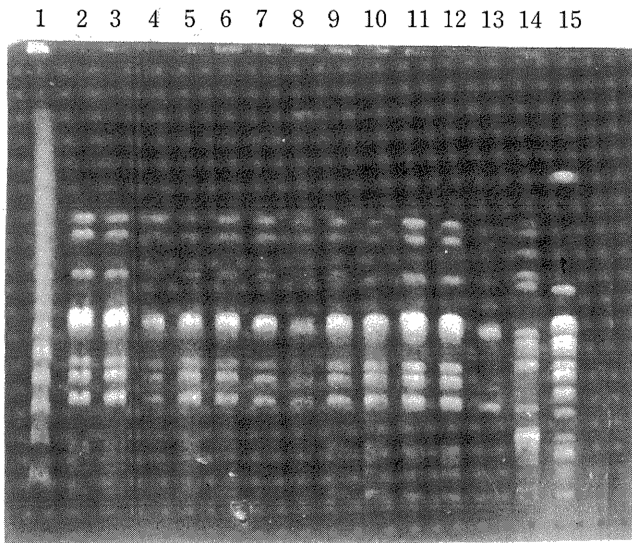

Figure 1 PFGE banding pattern of *Sfi*I-digested total cellular DNAs from *V. parahaemolyticus* isolates.
lane 1: Molecular mass maker (bacteriophage lamda DNA ladder): lanes 2, 3, 4: the strains 97LVP1, 97LVP2, 97LVP8 (Chanthabory Hospital); lanes 5, 6: the strains 97LVP23, 97LVP32 (Mahosot Hospital); lanes 7, 8, 9: the strains 97LVP24, 97LVP25, 97LVP28 (Sethatilath Hospital); lanes 10, 11, 12, 13: the strains 97LVP33, 97LVP34, 97LVP35, 97LVP36 (Sikhotabong Hospital); lane 14: the strain isolated from suspicious food (crab); lane 15: the strain isolated from a diarrhea patient in Laos in 1993.

observed to be very strong. However, the abilities of growth in 10% NaCl-peptone water and strong swarming disappeared within 6 months after isolation. The organisms were hemolytic on Wagatsuma medium with human erythrocytes, but hemolysis was variable when sheep erythrocytes were used. Serotypes were all O3:K6. However, an isolate from a minced crab which was suspected to be the causative agent did not grow in 10% NaCl-peptone water from the beginning, had a serotype of O2:K28, and was hemolytic. One isolate from a patient in 1993 did not grow in 10% NaCl-peptone water, the serotype was O4:K63, and was hemolytic. None of the isolates produced urease (Table 1).

Gene analysis: As examined by PCR, *tdh* was detected in all isolates from the patients but not in the isolate from the suspicious food, and *trh* was totally negative. Genomic DNA of the 12 strains from the patient isolates (2 to 4 strains from each hospital), a strain from the suspicious food and a strain from a patient in 1993 were analyzed by PFGE after *Sfi*I digestion. The latter 2 strains exhibited different digestion patterns from each other and from the patient strains.

Table 2 Distribution of drug susceptibilities

MIC(μg/ml)	OFLX	ABPC	TC	EM	CFDN
>100	0	0	0	0	0
100	0	0	0	0	0
50	0	3	0	0	0
25	0	27	0	0	0
12.5	0	6	0	17	0
6.25	1	0	0	18	30
3.13	33	0	0	1	6
1.56	2	0	9	0	0
0.78	0	0	27	0	0
0.39	0	0	0	0	0
≦0.2	0	0	0	0	0

Total=36 strains

Among the patient strains, 11 strains were identical while one strain from Sikhotabong Hospital had a different digestion pattern (Figure 1).

Drug susceptibilities: The MICs of each drug against the isolates from the patients were distributed in a narrow range, 3.13 μg/ml of OFLX, 25 μg/ml of ABPC, 0.8 μg/ml of TC, 6.25 μg/ml of EM, and 6.25 μg/ml of CFDN within the variation of one tube (Table 2). An isolate from the suspicious food and another isolated in 1993 were also included in this sensitivity pattern.

Discussion

Although there have been a few sporadic cases of *V. parahaemolyticus* enteritis in Laos (Insisiengmay *et al.,* 1997), this is the first recorded outbreak. The responsible pathogens may belong to the same clone with Calcutta strain from the viewpoint of recent epidemiological features. However, a strain out of 12 examined had a different PFGE pattern, and strains with various serotypes have been isolated in the past few years. These facts suggest that illness due to *V. parahaemolyticus* is not uncommon in Laos. Since the Calcutta strain seems to have high epidemic potential and according to Okuda *et al.,* it is genetically distinct from the *V. parahaemolyticus* O3:K6 strains isolated before 1994. What we have found as the phenotypical distinction from the other *V. parahaemolyticus* clones is the weak growth in 10% NaCl containing peptone water and production of a novel filamentous phage (unpublished data). However, these phenotypes can not explain the epidemic potential of the Calcutta strain.

An isolate from the suspicious food was O2:K28 serotype, *tdh* negative, *trh* negative, hemolytic on Wagatsuma medium, and did not grow in 10% NaCl-peptone water. Thus this food can not be identified as

the outbreak source. The reason why this isolate is hemolytic on Wagatsuma medium in spite of negative *tdh* and *trh* is unclear. Thermostable direct hemolysin (TDH) is believed to be a major cause of hemolysis on Wagatsuma medium (Kanagawa phenomenon). TRH (TDH-related hemolysin) producing strains are negative for the Kanagawa phenomenon (Honda *et al.*, 1990). Therefore, it is possible that an isolate from the suspicious food produces a novel hemolysin.

The results of PFGE revealed that the present outbreaks were not caused by a single clone of *V. parahaemolyticus*, therefore, it is possible that the causative food is not of a single origin. Although the genetic homology of the present strains to Calcutta strains and to *V. parahaemolyticus* O3:K6 isolated before 1994 were not examined in the present study, most of the isolated pathogens may belong to the same clone with Calcutta strain from the viewpoint of recent epidemiological features. The isolates from the present outbreaks were not distinguishable via drug susceptibilities because of nearly identical susceptibility level.

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Jpn. J. Trop. Med. Hyg., Vol. 26, No. 4, 1998, pp. 323-326

Research Note:

INCIDENCE OF FILARIASIS AS A CO-INFECTION IN MALARIA PATIENTS COMING FROM THAI-MYANMAR BORDER BETWEEN 1995-1997

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Received June 23, 1998/Accepted October 26, 1998

Abstract: Concomitant infection with malaria and filariasis is known to occur in animals and the co-infection appears to lessen the severity of malaria. We report here the incidence of co-infection with filariasis among 4,201 malaria patients admitted to the Bangkok Hospital for Tropical Diseases, Mahidol University, Thailand, between 1995 and 1997. There were eight patients (0.2%) with microfilariae (all *Wuchereria bancrofti*) in the peripheral blood smear. Four of the 8 patients had falciparum malaria and two patients among this group had cerebral malaria which responded to treatment without any long term sequelae. The rest four patients, three had vivax malaria while the last one had uncomplicated mixed infection of falciparum and vivax malaria. Filariasis was asymptomatic in all patients and cured with diethylcarbamazine. The eight patients resided along the Thai-Myanmar border, which is known to be endemic for both diseases. Our findings indicate the existence of co-infection of malaria and filariasis in Thailand, especially among patients from the Thai-Myanmar border. From this small number of patients, it is difficult to conclude that filariasis affects the severity of malaria. However, the data does emphasize that early diagnosis and early treatment of both infections is possible.

Key words: Filariasis, Malaria, Thailand

INTRODUCTION

Malaria and filariasis are mosquito-borne diseases. There are epidemiological studies in Southeast Asia about concomitant infection of malaria and filariasis in man (Kan *et al.*, 1985; Schuurkamp *et al.*, 1987). In Thailand, despite recent advances in vector control and chemotherapy, both of these infections remain major public health problems. The increasing number of Myanmar immigrants and the high incidence of the vector may lead to an epidemic of filariasis (WHO, 1997).

In 1997 the incidence rate of filariasis in Thailand is 2.08 per 100,000 (Surannadabba, 1996). The vector for filariasis is *Culex quinquefasciatus* which is found throughout Thailand. According to the Ministry of Public Health, Mae-Hong-Sorn, Tak, Kanchanaburi, Suratthani and Sarathiwat are five provinces in Thailand, which are endemic for both malaria and filariasis (Triteerapraparp, 1997). There are several reports on the effect of filariasis on malaria in animal models. In monkeys, Schmidt and Esslinger found that the co-infected group developed less severe malaria than the group with malaria infection alone (Schmidt *et al.*, 1981). Recently Yan and colleagues found that the co-infection of malaria and *Brugia pahangi* in mice protected them from developing severe malaria (Yan *et al.*, 1997). To our knowledge there has been no report from Thailand about co-infection of malaria and filariasis in man.

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Materials and Methods

The present study was conducted prospectively in the Bangkok Hospital for Tropical Diseases, Thailand between January 1995 and December 1997. Blood samples were collected from the malaria patients. Complete blood count, blood urea nitrogen, creatinine and liver function test evaluations were performed at the time of admission and repeated on day 7, 14, 21 and 28 after admission. Thick and thin blood films were sent for Field's stain for the detection of malaria parasite and microfilariae (Chularerk *et al.*, 1970). The patients were examined every 12 hr for both malaria parasites and microfilariae until the parasitemia became negative and then once daily until discharge. The patients who had filariasis were treated with diethylcarbamazine (DEC) 6 mg/kg/day for 6-12 day.

Results

There are 4,201 malaria patients admitted to the Bangkok Hospital for Tropical Diseases; 75.5% were male and 24.5% were female. 9.9% (418/4,201) were diagnosed as severe malaria (PF) and the rest were uncomplicated malaria both PV, PF. Eight of 4,201 malaria patients (0.2%) had co-infection with filarial parasites (Table 1). The mean (±SD) age of the patients was 27.4±5.6 years. All of them were male and lived along the Thai-Myanmar border. *Wuchereria bancrofti* was the only species found in blood smears. Four of the eight patients with filariasis had *P. falciparum*, three had *P. vivax* and one had mixed *P. falciparum* and *P. vivax*. Six of the eight patients had uncomplicated malaria infections and two patients had cerebral malaria. The clinical findings of the 8 patients are shown in Table 2.

All eight patients responded to treatment. Parasite clearance time (PCT) and fever clearance time (FCT) of *P. vivax*-infected patients were 45±19.3 and 18±14 hr respectively. PCT and FCT of *P. falciparum*-infected patients were 125.5±123.7 and 39.5±42.9 hr respectively. The initial geometric mean parasite counts of *P. vivax* and *P. falciparum* were 31,405 and 54,701/μl respectively. Patients no. 7 and 8 had cerebral malaria with

Table 1 Incidence of concomitant infections of malaria and filariasis of Bangkok Hospital for Tropical Diseases, Mahidol University, Thailand (1995-1997)

Year	No. of malaria infection	*P. falciparum* (Pf)	*P. vivax* (Pv)	*P. malariae* (Pm)	*P. ovale* (Po)	Mixed infection (Pf+Pv)	Mixed (malaria+ filariasis)
1995	1,363	656	646	9	0	52	3
1996	1,655	870	688	15	1	81	4
1997	1,183	583	551	6	1	42	1
Total 3 years	4,201 (100%)	2,109 (50.2%)	1,885 (44.9%)	30 (0.7%)	2 (0.05%)	175 (4.2%)	8 (0.2%)

Table 2 Details of 8 patients with co-infection of malaria and filariasis (1995-1997)

Patient No.	Sex	Age (years)	Previous malaria (No. of episodes)	Type of malaria	Severity of malaria	Initial parasites count (/μl)	PCT (hours)	FCT (hours)	Result of treatment	Other co-infection
1	M	30	0	Pv	Uncomplicated	10,100	67	34	Cure	Al, Hw, Tt
2	M	35	1	Pv	Uncomplicated	10,680	31	12	Cure	Ov, Ss, Tt
3	M	23	0	Pv	Uncomplicated	288,000*	37	8	Cure	None
4	M	30	7	Pv+Pf	Uncomplicated	9,480	65	298	Mix Pf D_{11}	Tuberculosis
5	M	23	0	Pf	Uncomplicated	4,020	309	0	R_2	Al, Tt
6	M	24	0	Pf	Uncomplicated	81,600	38	22	R_1D_{18}	Tt
7	M	35	0	Pf	Cerebral malaria	249,600*	79	100	R_1D_{24}	Hw, Ss, Tt
8	M	19	0	Pf	Cerebral malaria	109,200	76	36	R_1D_{21}	None

M=Male
Pv=*Plasmodium vivax*
Pf=*Plasmodium falciparum*
PCT=Parasite clearance time
FCT=Fever clearance time
R_1=Resistant type 1
R_2=Resistant type 2
Al=*Ascaris lumbricoides*
Hw=Hookwarm
Ov=*Opisthorchis viverrini*
Ss=*Strongyloides stercoralis*
Tt=*Trichuris trichiura*
*=Hyperparasitemia
D11=Day 11 after admission

Table 3 Clinical and laboratory findings of concomitant infections of malaria and filariasis of Bangkok Hospital for Tropical Diseases, Mahidol University, Thailand (1995-1997)

	Concomitant infections (malaria+filariasis) (n=8)
Sex M/F	8/0
Age (years)	
Mean±SD	27.3±5.6
Hematocrit (Mean±SD) (%)	29.7±8.6
Total eosinophil count Day 0 (Mean±SD) (/mm³)	168.8±236.7
G6PD normal/deficiency	8/0
Parasite clearance time (mean±SD) hr	
-*P. vivax* group	45±19.3
-*P. falciparum* group	125.5±123.7
Fever clearance time (mean±SD) hr	
-*P. vivax* group	18±14
-*P. falciparum* group	39.5±42.9
Geometric mean parasites count/μl (range)	
-*P. vivax* group	31,405(10,092-288,740)
-*P. falciparum* group	54,701(4,018-249,459)

G6PD=glucose 6 phosphate dehydrogenase

Glasgow coma scores of 7 and 8 respectively. Both of them were initially treated with a standard dosage of arteether but both recrudesced on day 24 and 21. One patient responded to further treatment with artesunate followed by mefloquine, and the other patient responded to quinine followed by tetracycline. One patient had hyperparasitemia and acute renal failure which required hemodialysis. Both patients recovered with no detectable sequelae. The three uncomplicated cases of *P. vivax* (patient no 1-3) were treated with chloroquine and primaquine as standard regimen and the three uncomplicated cases of *P. falciparum* were treated with quinine and tetracycline.

All patients with co-infection of malaria and filariasis were asymptomatic for filariasis. The average date of the positive smear for filariasis was the 9th day after admission and all patients were treated with diethylcarbamazine. The mean hematocrit and eosinophil count (±SD) on the day of admission were 29.7±8.6% and 168.8±236.7/mm³ respectively (Table 3). Cost of the patients had other parasitic infections; such as hookworm and *trichuriasis*.

DISCUSSION

In our study, of co-infection of malaria and filariasis was 0.2%. It was lower than the Indian study by Ravindran and colleagues which found a incidence of 0.33% (Ravindran *et al.*, 1998). Most of the patients in both studies came from a highly endemic area of filariasis and malaria. The differences of our study from previous work was that our data collected the patients who were admitted to the hospital. The clinical picture of our malaria infection was more severe than the community-based population.

The characteristic findings of the patients in our study confirm the results of a report by the Ministry of Public Health (Suvannadabba *et al.*, 1998) with respect to age, sex and geographical location. The eight patients of this study came from Kanchanaburi and Tak provinces where the endemicities of both malaria and filariasis, especially *W. bancrofti* infection, are still high. The PCT and FCT of the co-infection group were not different from the results of patients with malaria infection alone. However, the results may not be conclusive because of the small number of cases.

The majority of the patients with co-infection had uncomplicated malaria. There were two patients who had cerebral malaria. It seemed that our data did not go along with the previous data which demonstrated that the co-infection had protected the patients from developing severe malaria.

However we cannot conclude the reason of this finding because we did not study in the detail about the immune response of both cerebral malaria patients.

The hematocrit was low in the majority of the patients due to poor nutritional status and other parasitic infections such as hookworm. Most of the cases

had iron deficiency anemia. The average eosinophil count on the day of admission was $168 \pm 236.7/mm^3$ which was lower than average counts of $3{,}000/mm^3$ reported previously for filariasis (Triteerapraparp, 1997). However, all of our patients were asymtomatic for filariasis while the patients of previous reports of isolated filariasis were symptomatic with cough, fever and abnormal lung infiltration, which were related to hypersensitivity to microfilarial antigens.

The results of our study emphasize the existence of co-infection of malaria and filariasis in our patient population. Despite the low incidence we should be aware of this possibility. This finding should alert physicians to early diagnosis and early treatment of the co-infection in order to reduce disease transmission.

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ISSN 0304-2146

JAPANESE JOURNAL OF TROPICAL MEDICINE AND HYGIENE

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