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Sequence Analysis of Three Major Antigens (P30, P23 and P22) of Virulent and Avirulent Strains of *Toxoplasma gondii*

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ABSTRACT

We have sequenced the cDNA encoding the major surface antigens P30 and P22, and the excreted-secreted major antigen P23 of the virulent RH strain and avirulent Beverley and S-273 strains of *Toxoplasma gondii* by direct PCR. The amplified sequences of antigen cDNA were compared. Complete homology was found in the cDNAs encoding the P22 antigen in the RH and S-273 strains of *T.gondii*, and high homology but not complete was found between the cDNA encoding P23 and P30 in the Beverley and S-273. An interesting finding was the amino acid sequence differences relating to surface charge of P30 of virulent and avirulent strains of *T.gondii*. These phenomena might be associated with infectious difference in cells and/or the differences in pathogenicity.

INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite is an important ubiquitous pathogen in veterinary and human medicine. It is known to cause transplacental infections that can lead to abortion or severe neonatal malformation (Remington and Desmonts 1983). Congenital toxoplasmosis remains a major health problem in developed countries and also causes great economic loss in animal breeding, particularly sheep and pig farming. Acute toxoplasmosis has been also observed in patients immunocompromised as a result of drug therapy

(Cohen 1970), neoplastic disease (Frenkel et al. 1975), or infection (Luft et al. 1984) and is a cause of severe complications and death in AIDS patients (Navia et al. 1986). *Toxoplasma gondii* is a successful parasite of immunocompetent hosts because it readily forms cysts within brain and muscle tissue. These tissues are impervious to the immune system, which otherwise effectively controls acute infection and disease symptoms. This organism is considered an opportunistic pathogen because the cysts, which may exist for the life of the host, occasionally rupture and release thousands of highly invasive parasites which may cause a disseminated and potentially fatal disease if the host is in a state of immune deficiency. Therefore, the development of a vaccine against toxoplasmosis and specific knowledge on the host defense response would be of great value both in human and in veterinary medicine. Two major surface antigens, P22 and P30 (SAG1) that anchor the plasma membrane via a glycophospatidylinositol anchor, are expressed on the surface membrane of *T.gondii.* (Kasper et al. 1984; 1989, Tomavo et al. 1988, 1992; Nagel et al. 1989). Several investigators have produced monoclonal antibodies (mAb) which react specifically with the P30 antigen (Ag) (Kasper et al. 1992; Kim and Boothroyd 1993; Mineo and Kasper 1994; Bulow and Boothroyd 1991; Johnson et al. 1983; Rodriguez et al. 1985; Burg et al. 1988) and P23-24 Ag (Cesbron-Delauw 1989). A major excreted-secreted major antigen, termed P23, that has Ca binding domains, is suggested to be a posttranslationally processed product of the 24kDa polypeptide (P24) (Cesborn-Delauw et.al. 1989).

Here we report the comparison of the cDNA encoding P30, P23 and P22 of three different *T. gondii* strains.

MATERIALS AND METHODS

Toxoplasma gondii growth

Tachyzoite of the RH, Beverley and S-273 strains of *T. gondii* were grown in tissue culture using a HeLa cell monolayer in vitro culture system with modified Dulbecco's MEM + 10% heat inactive fetal calf serum (FBS) (Valkoun 1983). Purified parasites were obtained as previously described (Kato et al. 1994) and washed with ice cold phosphate buffer saline solution (PBS).

cDNA Synthesis

Approximately $5x10^7$ *T. gondii* and HeLa cells (negative control) were used for cDNA synthesis. Total RNA of each *T. gondii* strain was prepared using RNA Isolator (Genosys, Houston, USA). Messenger RNA (mRNA) was purified using BioMag Oligo (dT)20 (Nihon Perseptive, Tokyo, Japan). As a template for the amplification reaction, cDNA were prepared from 1mg of mRNA using Oligo

dT12 primer and 200U of Superscript II reverse transcriptase (Life Technologies, Tokyo, Japan) in 20ml of reaction mixture according to the manufactures's instructions. The cDNA reaction mixture was heated at 70℃, 15min and diluted to 100ml with TE buffer (10mM Tris-HCl, 1mM EDTA , pH 7.4) and stored -20℃ until use.

PCR Amplification of cDNA encoding antigens

All PCR primers were synthesized based on previously reported sequences (Prince et al. 1990; Cesbron-Delauw et al. 1989; Burg et al. 1988) using a Cyclone DNA synthesizer (Millipore Co., Tokyo, Japan) and purified on TSKgel Oligo DNA-RP HPLC column, 4.6x150mm (TOSOH, Tokyo, Japan). A nested PCR was performed for P30 cDNA amplification to improve specificity. For first step PCR, 100ml PCR reaction mixture containing 5ml of diluted cDNA reaction mixture as a template, 50pmol each primer (P30F1:AATGTGCACCTG TAGGAAGCTG,P30R2: GCACATGCTGCACGAAGTGTG), 0.2mM of each dNTPs, 10mM Tris-HCl (pH 8.85), 25mM KCl, 5mM $(NH_4)_2SO_4$, 2mM $MgSO_4$ and 2.5U of Pwo DNA polymerase (Boehringer Mannheim) was prepared. For the nested PCR step, 100ml PCR reaction mixture containing 5ml of first PCR amplified mixture as a template, 50pmol each primer (P30 S2nd:GTACAGTTTT TGTGGGCAGAGC, P30AS2nd: TTGTCGATTTGA-GAAGTGAGCA), 0.2mM of each dNTPs, 10mM Tris-HCl(pH 8.3), 50mM KCl, 1.5mM $MgCl_2$ and 2.5U of Taq DNA polymerase (Perkin-Elmer, Tokyo) was prepared. PCR reaction for P22 and P23 cDNA amplification was performed with 5ml of diluted cDNA reaction mixture and specific primers (P22Fd:ACAATTGCGGTGTGACACCTTC, P22Rv: GGTGCATATCTTGGTGTGACCT for P22 cDNA amplification, P23Fd: TGT-TTGGTGGCTGGCCAAATCA, P23Rv: GCAGGTGAAGTAACATGGGGTA for P23 cDNA amplification) in the same manner as P30 nested PCR reaction. All amplification reactions were performed as follows: denaturation, at 93℃ for 3min in the first cycle and for 1min in all 25 subsequent cycles: annealing, at 54℃ for 1min : extension, at 72 ℃ for 2min and for 10 min in the only last cycle on Quick Thermo II (Nippon Genetics, Tokyo, Japan). 10ml of PCR mixture was electrophoresed in 2% agarose gel and detected by ethidium bromide staining.

Purification of PCR Product

The PCR Reaction mixture was purified directly on TSKgel DNA NPR anion exchange column, 4.6x7.5cm (Elena D Katz et.al. 1990) using CCPM HPLC system and UV-8020 micro flow cell UV detector operated at 260nm (TOSOH) at 0.9ml/min flow rate. The 90μl of PCR production mixture were injected onto the column equilibrated with 55% of Buffer A (10mM Tris-HCl, 1mM EDTA pH9.0),

and 45% of Buffer B (Buffer A containing 1M NaCl). The eluant conditions were followed by 0.5min linear gradient from 45% to 55% and 12min gradient from 55% to 65%B. The 2.5 volumes of ethanol, 0.1 volumes of 3M Sodium acetate pH 5.2 and 10mg of glycogen were added into the purified fraction by HPLC. The purified products were allowed to precipitate at -20℃ for several hours and centrifuged at 15,000rpm 15min at 4℃. The pellet was washed with 1ml of 70% ethanol and dissolved in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 7.4).

Sequence Analysis

Sequences of all PCR amplified antigen cDNA were analyzed by the 373A automated DNA sequencer (Applied Biosystem, Tokyo, Japan) using the dye terminator cycle sequencing kit and several internal primers. The sequence data were analyzed and compared by Genetyx Mac and Geneworks program (IntelliGenetics, U.S.A).

RESULTS

The common laboratory strain, RH, is well known for its exceptional virulence in mice and its unusually rapid growth in vivo and in vitro. Therefore, it may not be an appropriate model for experimental infection and protection studies. The S-273 strain is a wild-type isolated from a infected pig in Japan. As it is capable of infecting pigs, shows the typical properties of slow growth in tissue culture, and, at low doses results in a nonlethal, chronic infection in mice with the appearance of brain cysts several weeks post infection, it is a typical *T. gondii* strain found in nature. To determine the homology between several cDNAs encoding major antigens of two avirulent and a typically virulent *T. gondii* strain we sequenced the P22, P23 and P30 cDNA from Beverley, S-273 strains and the RH strain. The sequences of the cDNA encoding P22, P23 and P30 of these strains are shown in Fig.1. Amino acid sequences translated from the cDNA sequences are shown in Fig.2.

The cDNA and amino acid sequence of the P22, P23 and P30 cDNA from the RH strain in our laboratory were identical with those of previous reports (Burg et al. 1988; Cesbron-Delauw et al. 1989; Prince et al. 1990).

There were a total of 14 nt differences among 1135 nt which resulted in 12 AA changes among 336 AA that differed between the RH and S273 strain P30 cDNA in the region sequenced. Nine AA of those , position #78 Phe-Ser, #195 Asp-Asn, #213 Lue-Phe, #251T-Ser, #263 Lys-Asn, #270 Lys-Asn,#298 Lys-Gln, #311 Ala-Ser, 325 Ile-Thr, were in the portion encoding mature peptide. The Sequence of the Beverley strain P30 cDNA revealed differences in 18 nt which resulted in 12 AA changes from that of the RH strain : position #67 Ala-Gly, #195

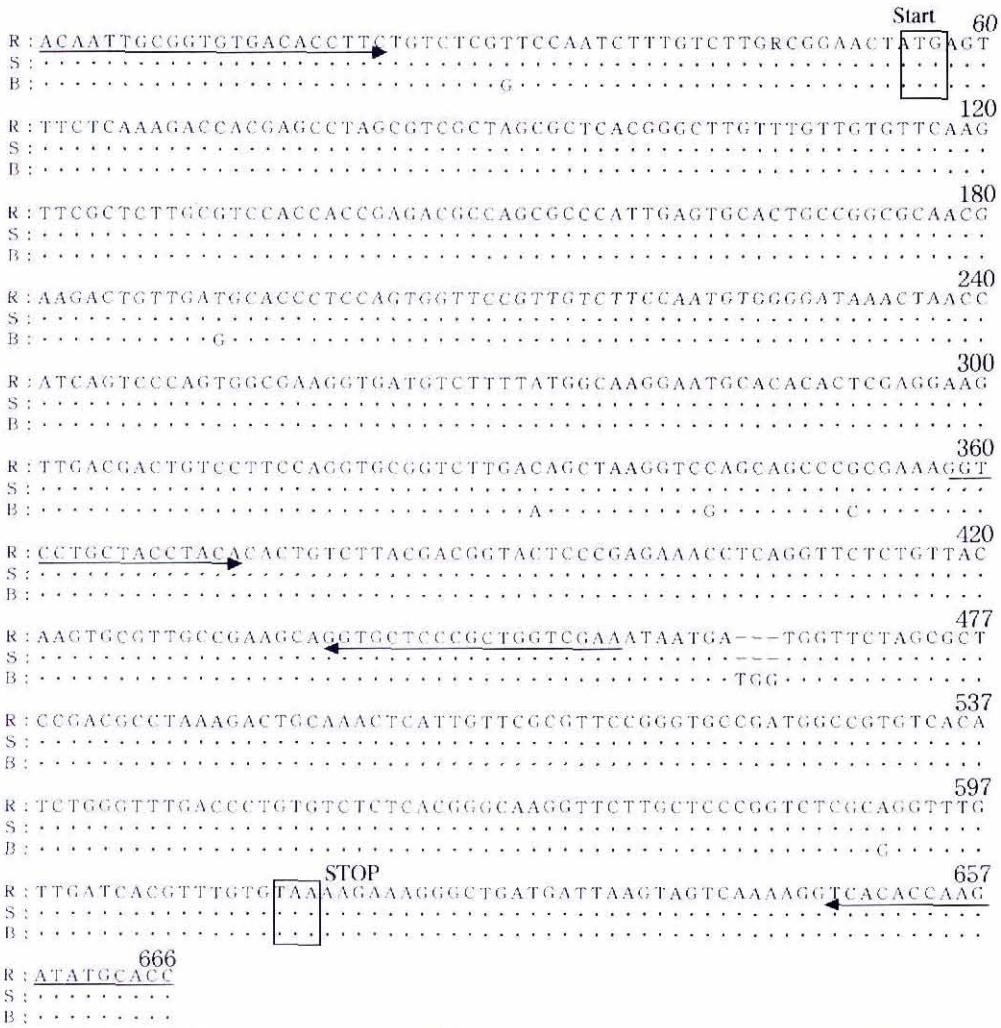

Fig. 1a cDNA Sequence comparison of P22 antigen. Arrows are showing primer site for PCR amplification and sequencing. R: RH strain, S: S-273 strain, B: Beverley strain.

Asp-Asn, #251Thr-Ser, #263 Lys-Asn, #270 Lys-Asn,#280 Ser-Gly, #298 Lys-Gln, #311 Ala-Ser, 325 Ile-Thr of the portion encoding mature peptides.

For the P22 cDNA comparison, no difference was observed between the RH strain and the S-273 strain, however, P22 cDNA between the RH strain and the Beverley strain differed in 6 nt and a 3nt insertion among 666 nt. As a result, 5 AA, position #46 Asp - Glu, #93 Thr-Lys, #97 Gln-Glu, #100 Ala-Pro in the common sequence and #138 Gly in the insertion occurred in the peptide of the Beverley strain.

The sequence of the P23 cDNA of the S-273 strain differed in 9 nt among 691 nt (which resulted in 6 AA differences among 190 AA) from that of the RH strain : position #42 Phe-Lue, #60 Asp-Glu, #86 Ala-T, #99 Val-Met, #114 Asp-

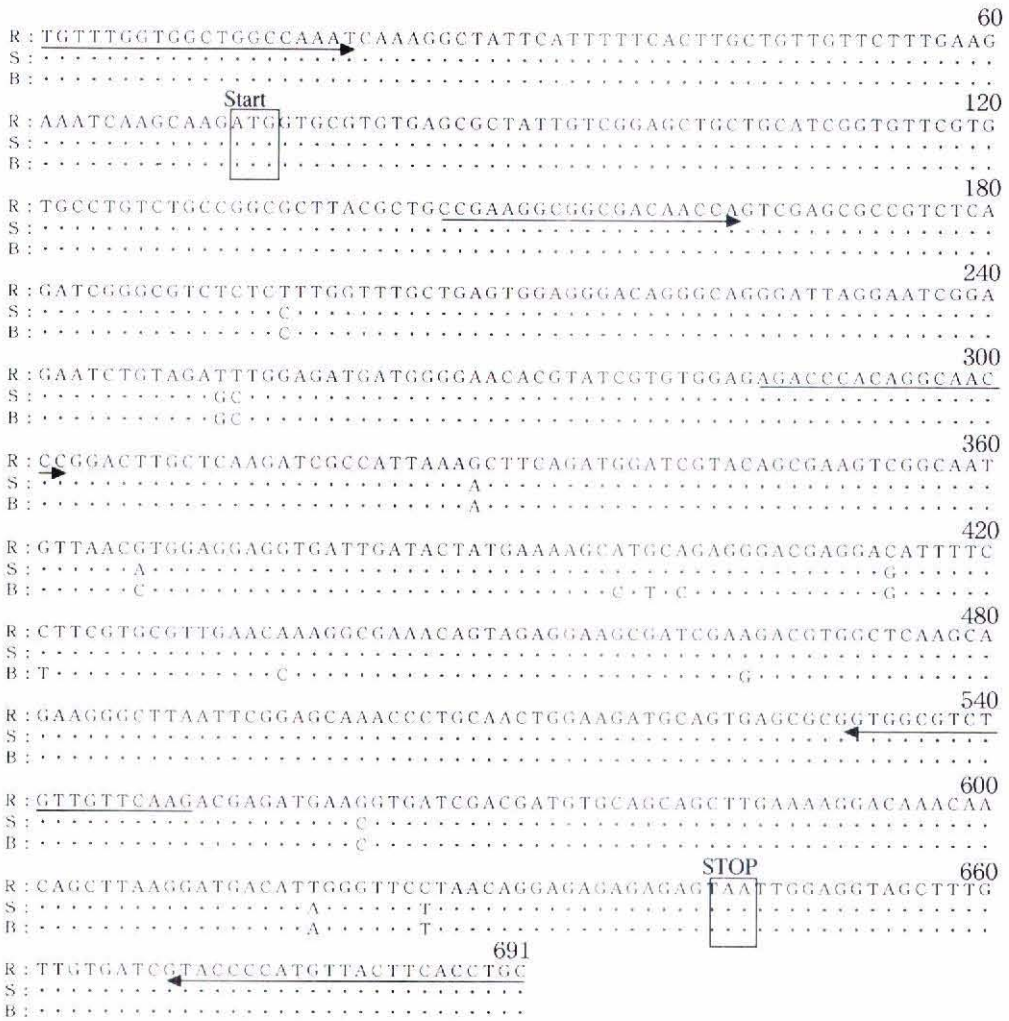

Fig. 1b cDNA Sequence comparison of P23 antigen. Arrows are showing primer site for PCR amplification and sequencing.

Glu, #163 Lys-Asn. There were a total of 15 nt among 691 nt (which result in 10 AA among 190 AA) that differed between the RH strain and the Beverley strain P23 cDNA (#42Phe-Lue, #60 Asp-Glu, #86 Ala-Thr, #99 Val-Lue, #109 Met-Lue, #110 Gin-Pro, #114 Asp-Glu, #117 Lue-Phe, #122 Lys-Gln, #163 Lys-Asn).

DISCUSSION

We describe here the sequence analysis of the cDNA encoding the major surface Ags (P30 and P22) and ES Ag (P23) of the RH, Beverley and S-273 strain of *T. gondii*.

The P30 and P23 cDNA sequences from the S-273 strain and the Beverley strain which are both avirulent *T. gondii* were observed in high similarity and homology. An interesting finding in two of the three strains studied was the

Fig. 1e cDNA Sequence comparison of P30 antigen. Arrows are showing primer site for PCR amplification and sequencing.

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                                                                60
R : MSFSKTTSLASLALTGLFVVFKFALASTTETPAPIECTAGATKTVDAPSSGSVVFQCGDK
S : ............................................................
B : .............................................E..............
                                                               120
R : LTISPSGEGDVFYGKECTDSRKLTTVLPGAVLTAKVQQPAKGPATYTLSYDGTPEKPQVL
S : ............................................................
B : ................................K...E..P....................
                                                               180
R : CYKCVAEAGAPAGRNND-GSSAPTPKDCKLIVRVPGADGRVTSGFDPVSLTGKVLAPGLA
S : .................–..........................................
B : .................G..........................................
        187
R : GLLITFV
S : .......
B : .......
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Fig. 2a Amino Acid Sequence comparison of P22 antigen. Signal peptide is underlined. Arrows Show the residue that changes the ionic charge. Inserted Gly are boxed.
R: RH strain, S: S-273 strain, B: Beverley strain.

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                                                                60
R : MVRVSAIVGAAASVFVCLSAGAYAAEGGDNQSSAVSDRASLFGLLSGGTGQGLGIGESVD
S : .........................................L.................E
B : .........................................L.................E
                                                               120
R : LEMMGNTYRVERPTGNPDLLKIAIKASDGSYSEVGNVNVEEVIDTMKSMQRDEDIFLRAL
S : .........................T............M..............E......
B : .........................T............L.........LP...E..F...
                                                               180
R : NKGETVEEAIEDVAQAEGLNSEQTLQLEDAVSAVASVVQDEMKVIDDVQQLEKDKQQLKD
S : ..........................................N.................
B : .Q........................................N.................
           190
R : DIGFLTGERE
S : ..........
B : ..........
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Fig. 2b Amino Acid Sequence comparison of P23 antigen. Signal peptide is underlined. Ca^{2+} binding domains are boxed. Arrows show the residue that lost anionic charge.

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                                                                60
R : MSVSLHHFIISSGFLTSMFPKAVRRAVTAGVFAAPTLMSFLRCGVMASDPPLVANQVVTC
S : ................A...R........................A..............
B : ...............WA............................A..............
                                                               120
R : PDKKSTAAVILTPTENHFTLKCPKTALTEPPTLAYSPNRQICPAGTTSSCTSKAVTLSSL
S : .................S..........................................
B : ......G.....................................................
                                                               180
R : IPEAEDSWWTGDSASLDTAGIKLTVPIEKFPVTTQTFVVGCIKGDDAQSCMVTVTVQARA
S : ............................................................
B : ............................................................
                                                               240
R : SSVVNNVARCSTGADSTLGPVKLSAEGPTTMTLVCGKDGVKVPQDNNQYCSGTTLTGCNE
S : ..............N.................F...........................
B : ..............N.............................................
                                                               300
R : KSFKDILPKLTENPWQGNASSDKGATLTIKKEAFPAESKSVIIGCTGGSPEKHHCTVKLE
S : ..........S...........N......N...........................Q..
B : ..........S...........N......N.........G.................Q..
                                    336
R : FAGAAGSAKSAAGTASHVSIFAMVIGLIGSIAACVA
S : ..........S.............T...........
B : ..........S.............T...........
```

Fig. 2c Amino Acid Sequence comparison of P30 antigen. Signal peptide is underlined. Avirulence strain specific glycosylation site is boxed. Arrows Show the residue that lost anionic charge.

change in the same AA in #263 and #270 Asn and #298 Gln, while the RH strain was Lys, which may result in a reduction in the molecular surface positive charge in this region. On the other hand, the change of #195 Asp-Asn found in the S-273 and Beverley strains resulted in an Asn-X-Thr composition, which may result in glycolization of the #195 Asn. P30 has been reported as an important parasite ligand involved in the process of attachment to host cells (Mineo et al. 1994), and this difference in amino acid sequence may be associated with the infection and pathogenicity of *T. gondii*. Several observations suggested that P30 is a complex protein with considerable higher order structure and multiple forms (Handman and Remington 1980; Kasper 1987; Rodrigues et al. 1985; Santoro et al. 1985; Lawrence-Burg et al. 1988).

Here, we have confirmed that the major surface Ags P30 and P22, and ES Ag P23 of T.gondii are appropriate for further studies producing transgenic animals, which will be extremely valuable to examine the host immune mechanisms against Toxoplasma infection.

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