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Production of Transgenic Mice Carrying p30 Gene Encoding Major Surface Antigen of *Toxoplasma gondii*

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ABSTRACT

To generate transgenic mice carrying a protozoan gene, 3.1 kb DNA fragments carrying the CAG promoter ligated with a protozoan gene encoding a major surface antigen SAG-1 (p30) of *Toxoplasma gondii*, were microinjected into one of pronuclei of embryos of C57BL/6J and BALB/c mice. The embryos were transferred to the oviducts of ICR pseudopregnant recipients. The transgene was detected by polymerase chain reaction in DNA sequence purified from tissue biopsies. Out of 159 mice that developed from injected eggs two p30-founders (BALB/c and C57BL/6J) were obtained. The transgene was not detected in 52 pups of F1 progeny of C57BL/6J founder. However, 3 (17.6%) out of 17 F1 progenies from BALB/c founder were found to have p30 gene and they inherited the transgene to 39 (55.7%) of 70 F2 progenies. These results may afford the opportunity to study the role of SAG-1(p30) gene in *Toxoplasma gondii* infection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite

responsible for toxoplasmosis that infects most species of warm-blooded animals including domestic animals, bird and human, in most parts of the world. The disease has been known to result in blindness, mental retardation and death of congenitally infected infants (Dubey 1977; McCabe 1970) and AIDS patients (Mills 1986).

Mammalian cells can be infected by the parasite in vitro, although intracellular growth occurs principally within nucleated cells (Joiner and Dubremetz 1993; McLeod et al. 1991; Buckley 1973). Attachment to the host cell is the first step required in the process of invasion. SAG-1(p30), the major surface protein of *T. gondii* (Kasper and Boothroyd 1993), is involved in the process of host cell infection (Mineo et al. 1993; Grimwood and Smith 1992; Robert et al. 1993) and is considered as an important ligand for binding to the host cell in the process of *T. gondii* invasion (Mineo and Kasper 1994).

It has been shown that the susceptibility to *T. gondii* infection varied among inbred mouse strains; BALB/c, with Ld gene has lower cyst burdens and less encephalitis than those, C57BL/10J, without the Ld gene (Brown et al. 1995). Mice that were resistant to *Toxoplasma* infection had little detectable cytokine mRNA expression in brain, while mice that were susceptible had elevated levels of mRNA for a wide range of cytokines, consistent with their greater amounts of inflammation (Brown et al. 1995). So far, there have been no report on the generation of transgenic animals carrying protozoan DNA, although Moleon et al. (1994) described the cloning of p30 gene by PCR for the purpose of transgenic mice production.

The purpose of this study was to generate transgenic mice carrying p30 gene of *T. gondii* protozoan parasite using BALB/c and C57BL/6J mice in order to analyze the role of p30 in *T. gondii* infection on different genetic background.

MATERIALS AND METHODS

In Vitro Fertilization

BALB/c and C57BL/6J mice, 8 weeks of age, purchased from a commercial supplier, CLEA, Japan,Inc., were superovulated by i.p. injection of 5 I.U of pregnant mare's serum gonadotropin (PMSG; Sankyo Zoki Co., Japan) and human chorionic gonadotropin (hCG; Sankyo Zoki Co.) with an interval of 48 hrs. In vitro fertilization and embryo culture were performed at 37 °C in a 5% CO_2 in air atmosphere. Unfertilized eggs were collected from oviducts of treated mice approximately 16 hrs post hCG injection into a drop of 400 μl of the fertilization medium (TYH) (Toyoda et al. 1971). A small amount of 2 hrs-preincubated sperm suspension was introduced to TYH medium containing cumulus-enclosed oocytes.

Approximately 5 hrs after insemination, fertilized eggs were harvested and washed several times and transferred to a microdrop of 100 μl of Whitten's medium (Whitten 1971) supplemented with 100 μM ethylenediaminetetraacetic acid (EDTA) (Hoshi and Toyoda 1985).

DNA Preparation for Microinjection

p30 gene was cloned from the cDNA obtained by RT-PCR amplification of the *T.gondii* mRNA according to the published sequence of the gene (Burg et al. 1988) as described elsewhere (Maki et al. 1996). A plasmid pexCANLacZ (Kanegae et al. 1995), that has been used for successful generation of LacZ-expressing transgenic mice (Tsukui et al. 1996), was digested with ClaI and the p30 gene was inserted to the ClaI site instead of LacZ. The plasmid pCAGP30, thus constructed, was digested with SalI and HindIII and the expression unit fragment containing CAG (Cytomegarovirus enhancer-chicken β-actin hybrid) promoter (Niwa et al. 1991) and p30 gene was isolated on TSKgel DNA-NPR anion exchange column, 4.6×7.5cm, using a CCP&8020 HPLC system (TOSOH, Japan) and precipitated in ethanol. Purified fragment was dissolved in 10mM Tris-HCl, 0.1mM EDTA solution (pH 7.4) at a concentration of 10 mg/ml.

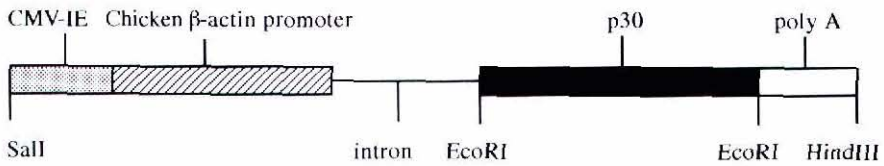

Fig.1 p30 Transgene Construct. The p30 transgene contains the CMV-IE enhancer, the chicken β actin promoter, p30 and rabbit β-globin poly A.

Pronucleous Microinjection

Pronuclear microinjection (Gordon et al. 1980) was performed under Hoffman modulation contrast optics on an inverted microscope (Nikon, Japan) armed with a micromanipulator (Narishige), and in Hepes-buffered mWM at approximately 10 hrs post insemination. One of visible pronuclei of the fertilized eggs was microinjected with the prepared DNA solution. Volume of the DNA solution injected into each pronucleus was estimated to be 2 pl (Allen et al. 1987). The injected embryos were washed with fresh medium and then cultured to the

two-cell stage in 100 μM EDTA supplemented Whitten's medium. The embryos were transferred to the infundibulum of oviducts of 0.5 day pseudopregnant ICR (CLEA, Inc., Japan) recipients.

Preparation of DNA

Distal 1 cm of tail from 4 weeks old mice under anesthetization with diethyl ether was excised with a sterile scissors. Tail biopsies were placed in 1.5 ml microcentrifuge tubes and digested for 4 hrs to overnight at 55 °C with 700 μl of digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, and 1 % sodium dodecyl sulfate) and 35 μl of proteinase K (10 mg/ml). The digests were extracted with 700 μl of TE-satured phenol, 700 μl of phenol/ chloroform, and 700 μl of chloroform. DNA was precipitated from the aqueous phase with 2.5 volumes of absolute ethanol and 1/10 volumes of 3M sodium acetate. Precipitates were washed with 70 % ethanol and air-dried. The pellets were then redissolved in 100 μl of sterile water.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in 50 μl total volume containing 250 ng of isolated DNA. The p30 gene was amplified using oligonucleotide primers consisting of 20 and 21 base pairs and corresponded to nucleotides 544-564 (cactctcaagtgccctaaaa) and 960-939 (gaactttgactccatctttcc). The PCR product was a 416 base pair fragment. The 50 μl reaction mixture contained a final concentration of 25mM $MgCl_2$, 2 mM of each dNTP, 1.6 units of Taq polymerase (Perkin Elmer) and 50 pmol of each oligonucleotide primer. Amplification was performed in an automated thermocycler (GeneAmp PCR system 2,400, Perkin Elmer). The condition for temperature cycling were: 94 °C for 5 min followed by 40 cycles of 94 °C for 20 sec, 53 °C for 30 sec, and 72 °C for 1 min. Cycling was followed by a final extension step at 72 °C for 10 min, and the reactions were held at 4 °C. Amplification products were then electrophoresed on a 1.5 % agarose gel, stained with ethidium bromide, and photographed under UV illumination.

RESULTS

Among 159 pups at 4 weeks of age, 32 BALB/c and 127 C57BL/6J, that had been weaned and were analyzed by polymerase chain reaction (Fig. 2A), two p30-founders, 1 BALB/c female and 1 C57BL/6J male, were identified. The success rate of the founder generated was 1.2 % (Table 1).

The p30-founders were bred with littermates and also with wild type mice of the same strain. The resultant F1 progeny was screened for the exogenous p30

Table 1 Success rate of generating p30-founder mice.

Mice	Number of weaned pups resulted from microinjection	p30-founder number	p30-founder rate (%)
BALB/c	32	1	3.1
C57BL/6J	127	1	0.7
Total	159	2	1.2

transmission by PCR (Fig. 2B).The exogenous gene was not detectable in 52 pups from C57BL/6J founder line (Table 2). For the BALB/c founder line, however, the p30 gene was detected in 3 of 17 pups, corresponding to 17.6% of transmission rate (Table 2). The mice were mated to obtain homozygous F2 progeny. PCR results (Fig. 2C) showed that 39 (55.7%) out of 70 pups were inherited the transgene from parents (Table 3).

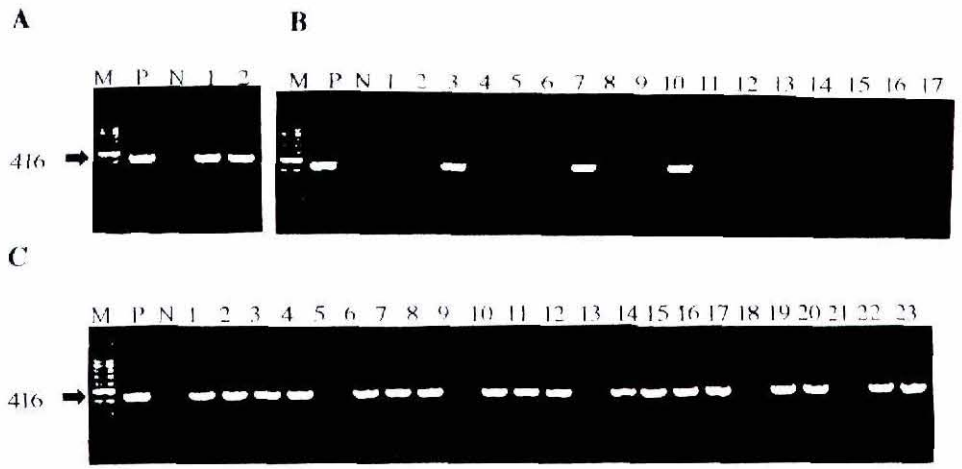

Fig. 2 Identification of p30 transgene by PCR. Mouse genomic DNA purified from tail biopsy amplified with oligonucleotide primers S-544 and AS-960. PCR products: 416 bp. The product (10 μl) were loaded onto a 1.5% agarose gel and visualized by ethidium bromide staining. A: DNA of mice resulted from microinjection, Lane 1: BALB/c, Lane 2: C57BL/6J. B: DNA derived from BALB/c F1 progeny, Lane 1-17. C: DNA derived from BALB/c F2 progeny of trangenic F1 male (No. 3), Lane 1-23. M: marker, P: positive control (microinjected DNA), N: negative control (DNA isolated from normal mouse).

Table 2 p30 transmission to F1 progeny.

p30-founder	Number of weaned progeny	Gene transmission	
		number	rate (%)
Female BALB/c	17	3	17.6
Male C57BL/6J	52	0	0.0

Table 3 p30 transmission to F2 BALB/c mice.

Transgenic F1 mice	Number of F2 pups	Gene transmission	
		number	rate (%)
Male No. 3	28	18	64.2
Male No. 7	33	16	48.4
Female No.10	9	5	55.5
Total	70	39	55.7

DISCUSSION

Introduction of the exogenous p30 transgene into fertilized eggs of BALB/c and C57BL/6J mice at pronucleus stage resulted in a p30-founder mouse of each strain. However, none out of 52 pups derived from C57BL/6J male founder was shown to have the transgene. Investigation on the C57BL/6J founder incapable of transferring genetically the p30 gene to his offspring was carried out. The transgene was detected in oral epithelial cells, tail and testis biopsies, but not in sperms recovered from uterine horns after being mated (data not shown). It stood reason for the founder incapable of transferring the gene to the progeny; and spermatogenesis might be involved. On the other hand, approximately 18% (Table 2) and 56% (Table 3) of progeny F1 and F2, respectively, derived from the BALB/c female founder were identified to inherit the p30 transgene. It indicated that the transgene was stably transmitted in BALB/c mice line. As far as, the authors are aware this is the first report of generation of transgenic mice carrying protozoan gene. Studies on the p30 gene product expression and its function in *Toxoplasma gondii* infection are being conducted.

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REFERENCES

Allen, N. D., Barton, S., Surani, M. A. H. & Reik, W. 1987. Production of transgenic mouse, pp 217-233, *In:* Mammalian Development. Monk, M (ed). Oxford, IRL Press.

Buckley, S. M. 1973. Survival of *Toxoplasma gondii* in mosquito cell lines and establishment of continuous infection in Vero cell cultures. *Exp. Parasitol. 64:* 474-484.

Burg, J. L., Perelman, D., Kasper, L. H., Ware, P. L. & Boothroyd, J. C. 1988. Molecular analysis of the gene encoding the major surface antigen of *Toxoplasma gondii. J. Immunol. 141:* 3584-3591.

Brown, C. R., Hunter, C. A., Estes, R. G., Beckmann, E., Forman, J., David, C., Remington, J. S. & Mcleod, R. 1995. Definitive identification of a gene that confers resistance against *Toxoplasma* cyst burden and encephalitis. *Immunology 85:* 419-428.

Dubey, J. P. 1977. *Toxoplasma, Hammondia, Besnoitia, Sarcosystis,* and other tissue cyst-forming coccidia of man and animals. p.166, *In:* Parasitic Protozoa. Vol. 3. J. B. Kreier, (ed). Academic Press, New York.

Furtado, G. C., Slowik, M., Kleinman, H. K., & Joiner, K. A. 1992. Laminin enhances binding of *Toxoplasma gondii* tachyzoites to J774 murine macrophage cells. *Inf. Immun. 60:* 2337-2342.

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Basrobosa, J. A. & Ruddle, F. H. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA 77:* 7380-7384.

Grimwood, J., & Smith, J. E. 1992. *Toxoplasma gondii:* The role of 30 KDa surface protein in host cell invasion. *Exp. Parasitol. 74:* 106-111.

Joiner, K. A. 1991. Cell attachment and entry by *Toxoplasma gondii. Behring Institute Mitteilungen 88:* 20-26.

Joiner, K. A. & Dubremetz, J. F. 1993. *Toxoplasma gondii:* A protozoan for the ninties. *Inf. Immun. 61:* 1169-1172.

Mineo, J. R. & Kasper, L. H. 1994. Attachment of *Toxoplasma gondii* to host cells involves major surface protein, SAG-1(P30). *Exp. Parasitol. 79:* 11-20.

Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. & Saito, I. 1995. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic.*

Acids. Res. 23: 3816-3821.

Kasper, L. H. & Boothroyd, J. C. 1993. *Toxoplasma gondii:* Immunology and molecular biology. pp. 269-301, *In:* Immunology and Molecular Biology of Parasitic Infections. Warren, K. S. (Ed), Cambridge. MA.

McCabe, R. E. & Remington, J. S. 1983. The diagnosis and treatment of toxoplasmosis. *Eur. J. Clin. Microbiol. 2:* 95.

McLeod, R., Mack, D. & Brown, C. 1991. *Toxoplasma gondii:* New advance in cellular and molecular biology. *Exp. Parasitol. 72:* 109-121.

Mills, J. 1986. *Pneumocystis carinii* and *Toxoplasma gondii* infection in patients with AIDS. *Rev. Infect. Dis. 8:* 1001.

Mineo, J. R., McLeod, R., Mack, D., Smith, J., Khan, I. A., Ely, K. H. & Kasper, L. H. 1993. Antibodies to *Toxoplasma gondii* major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after per-oral infection. *J. Immunol. 150:* 3951-3964.

Moleon, I., Beuchamp, P., Cesbron, F. M. & Tourvieille, B. 1994. Cloning of the gene encoding the p30 antigen of *Toxoplasma gondii* by PCR to insert it in transgenic mice. *Arch. Med. Res. 25:* 463-466.

Niwa, H., Yamamura, K. & Miyazaki, J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene 108:* 193-199.

Takeda, S. & Toyoda, Y. 1991. Expression of SV40-lacZ gene in mouse preimplatation embryos after pronuclear microinjection. *Mol. Rep. Del. 30:* 90-94.

Toyoda, Y., Yokoyama, M. & Hoshi, T. 1971. Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. *Jpn. J. Anim. Reprod. 16:* 147-151.

Toyoda, Y. 1995. In vitro culture and transfer of mammalian early embryos. Protein, nucleic acid and enzyme, Journal 40: 14 (in Japanese).

Tsukui, T., Kanegae, Y., Saito, I. & Toyoda, Y. 1996. Transgenesis by adenovirus-mediated gene transfer into mouse zone-free eggs. *Nature Biotechnol. 14:* 982-985.

Whitten, W. K. 1971. Nutrient requirements for the culture of preimplantantion embryo in vitro. *Adv. Biosci. 6:* 129-139.