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SUSCEPTIBILITY OF MICE TO *TOXOPLASMA GONDII* AFTER NUCLEIC ACID IMMUNIZATION

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SUMMARY

In the present study, mice were vaccinated with *Toxoplasma gondii* DNA vaccine and anti-toxoplasma antibodies were produced against the major surface antigen P30. Five mice (Balb/c) were vaccinated I/M with naked toxoplasma DNA vaccine (pGFP-N3 with p30 insert), five mice with pGFP-N3 without insert, four mice with pGFP-N3 with p30 insert complexed with cationic liposomes (DOTAP), four mice with pGFP-N3 without p30 insert complexed with cationic liposomes (DOTAP). Two Control groups were vaccinated with crude toxoplasma antigen and PBS. One group was kept as non injected control. Anti-toxoplasma antibodies were monitored serologically in the vaccianted mice by IFAT and ELISA. There was no difference in IFAT antibody response between the mice immunized with DNA vaccien and those immunized with DNA carried on cationic liposomes. All control mice challenged with 5 X10^4 live *T. gondii* tachyzoites died while those immunized with *T. gondii* DNA appeared to survive longer.

INTRODUCTION

Toxoplasma gondii was first discovered by Nicolla and Manceaux in 1908 in north african rodents. The cats and wild felidae act as definitive hosts while a wide range of warm blood animals can serve as intermediate hosts. Toxoplasmosis is an important zoonotic disease. It is estimated that one-third of the world population have been exposed to the parasite (Kean, 1972). The three well established mode of transmission of *T. gondii* are carnivorism, congenital and fecal contamination. In humasns, infection is acquired orally through

ingestion of uncooked infected raw meat and transplacentally (Beverley, 1959).

Asao and Akio (1991) reported that immunochemical analysis of the surface antigen of *T.gondii* has identified four major radioiodinoatable proteins which were named p43, p35, p30 and p22 according to their apparent molecular weight. The p30 or SAG1, a major surface antigen of *T. gondii,* is a highly imunogenic protein which has generated great interest as a diagnostic reagent, as a potential subunit vaccine and for its role in invasion. The antigen is abundant and is homogeneously distributed on the surface of both extracellular and intracellular trophozoites.

The p30 antigen is a 34.7 kDa protein and is encoded by a 1.5 kb single copy gene which contains no introns. The primary translation product has a carboxy-terminal hydrophobic tail which is predictive of a post-translational cleavage and modification with a glycolipid anchor. (Burg et al., 1988). Kim et al. (1994) reported successful conformationally appropriate expression of p30 in CHO cells from a *MboII-AvaI* fragment consisting of a 960 bp coding sequence and a 310 bp 3' untranslated region.

Nucleic acid vaccination or DNA vaccination refers to the direct use of nucleic acids (DNA) to stimulate an immune response. The recent develepomnt of vaccines based on nucleic acids is of particular interest because of their capacity to generate humoral and cellular immune response (Schodel et al., 1994). Also immunization with purified genetic material has some distinct advantages, it allows presentation of the parasitic antigen to the immune system in a native form. This study is a preliminary attempt to try the method of DNA vaccination and to induce antibodies in Balb/c mice against *T. gondii* major surface antigen, p30, by using a DNA vaccine consisting of a p30 sequence cloned into a eukaryotic expression vector.

MATERIAL AND METHODS

T. gondii Rh strain maintained in Balb/c mice at the Institute for Medical Research (Malaysia) was used for preparation of crude antien, extraction of DNA and for challenge of immunized mice with DNA vaccine. Mice were inoculated intraperitoneally with more than 10^5 viable tachyzoites resuspended in 0.9% sterile normal saline with penicillin (1000 U/ml) and streptomycin (100 μg/ml) and observed daily. Infected mice (showing ruffling of fur and reduced activity) were sacrified. The peritoneal contents were flushed out using 5 ml sterile 0.9% saline. The peritoneal fluid harvested from the Toxoplasma infected mice was filtered once through glasswool to remove contaminating peritoneal cells. The filtered fluid was then washed 3 times with normal saline and centrifuged at 3400 rpm for 10 minutes. The pellet containing *T. gondii* was resuspended in 1 ml lysis buffer [10 mM Tris HC1 (pH 8.0), 150 mM

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NaCl, 10 mM EDTA, 2% SDS] and incubated at 70°C for 10 minutes to destroy contaminating nucleases. After the suspension was cooled to room temperature, 50 µl of proteinase K (GIBCO BRL, 10 mg/ml) was then added to the mixture and incubated overnight at 55°C in a shaking incubator. The following day, an equal volume of phenol was added, mixed by inversion for 10 minutes and centrifuged at 2400 rpm for 15 minutes at room temperature. The supernatant was transferred into a new tube where an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (v/v/v) was added, mixed for another 10 minutes and centrifuged at 2400 rpm for 15 minutes at room temperature. the supernatant was transferred into another new tube and the same procedure repeated using an equal volume of chloroform: isoamyl alcoho (24:1) (v/v). The supernatant was then collected into a new tube and mixed with 1/10 volume of sodium acetate 3M. 2.5 volume of cold absolute alcohol was gently poured down the side of the tube. DNA was spooled out from the interface using a sterile glass pasteur pipette. The spooled DNA was rinsed with 70% ethanol and air dried. The DNA was reconstituted in 200µl Tris EDTA [10 mM Tris (pH 9.0), 1 mM EDTA]. The DNA left at 4°C for 3 days and diluted 1:20. The diluted DNA was analyzed with ethidium bromide stained agarose gel electrophoresis and quantitated by visual comparison of the band inteensity with DNA Mass Ladder (GIBCO BRL) and by spectrophotometry (Maniatis et al., 1989). The nucleic acid vaccine is 1.5 Kb single copy gene which contains no introns and encoding the p30 protein antigen which is the main surface immunogenic protein antigen of *T. gondii*. This 1.5 Kb insert was prepared by PCR using the forward primer (AACAGAAGATCTATGTCGGTTTCGCTGCACCACTTCA) and the reverse primer (GGGTCACGCGACACACAAGCTGCGATAGAGCC). The PCR products were digested with Bgl II - Pst I and ethanol precipitated. The digested PCR products were then ligated into plasmid pGFP-N3 (Clontech) and used to transform E.coli strain DH5°C by electroporation (Bio-Rad). The cloned P30 insert was used as vaccine.

Immunization of Balb/c mice with Toxoplasma nucleic acid vaccine.

Thirty three, four week old Balb/c mice were assigned to the following groups:

- Five mice immunized intramuscularly with naked toxoplasma nucleic acid vaccine (pGFP-N3 with p30 insert).
- Five mice immunized intramuscularly with naked control (pGFP-N3 without insert).
- Four mice immunized intramuscularly with toxoplasma nucleic acid vacine (pGFP-N3 with p30 insert) complexed with cationic liposomes (DOTAP).
- Four mice immunized intramuscularly with control (pGFP-N3 without insert) complexed with cationic liposomes (DOTAP).
- Five mice immunized intramuscularly with 200

µg crude antigen of *T. gondii* (control).

- Five mice immunized with PBS (control).
- Five mcie unimmunized (control).

DNA vaccine was injected through the skin of mice by fusion with N- {1- (2,3-Dioleoyloxy) propyl} -N, N,N-trimethylammonium methylsulfate (DOTAP) (Boehringer Mannheim) which is a cationic liposome formulation for transfection of DNA, oligonucleotides, ribonucleoprtein particles and protein into eukaryotic cells. It is able to produce efficient transfection in the presence of serum and has been successfully used for in vivo transfection (Boehringer Mannheim Product Information Cat. No. 1202375). A total of 50 µl of DNA solution (1 mg/ml) was used to give a total of 50 µg of DNA. For cationic liposome complexed DNA, a total of 50 µg of DNA was used in 250 µl of solution and injected I/M as two doses of 125 µl to each hind leg. the mice were immunized on weeks 0 and 2, blood were obtained on 0, 2 week post vaccination, another samples were obtained 2 week post boostering.

Preparation of *T. gondii* positive control.

16.6 µl toxoplasma crude antigen (3 mg/dl) was mixed with 3 ml complete Freund's adjuvant and 3 ml sterile normal saline. 4 week old Balb/c mice were injected intramuscularly with 2 ml of the mixture at weekly intervals for a total of 3 doses. Blood was collected on the fourth week and used as positive control. Negative control was obtained from unimmunized healthy Balb/c mice.

Preparation of crude antigens.

Rh strain of *T. gondii* crude antigen was prepared as for the preparation of DNA. The washed parasites was subjected to repeated freezing (for several minutes) in liquid nitrogen alternating with thawing at room temperature. The antigen was manually homogenized in ice, then sonicated in ice for 20 minutes and checked microscopically. The antigen was left over night at 4°C then centrifuged at 14,000rpm for 10 minutes. The supernatant was collected, estimated with BIO-RAD Protein Assay and stored at -20°C till further use.

Optimization of ELISA:

200 µl of 0.5ug/ml crude antigen in carbonate buffer (pH 9.6) was used to coat the ELISA microtitre plate wells. The ELISA was optimized using a chequerboard. The optimum concentraiton of goat anti-mouse Igs horseradish peroxidase conjugate (American qualex, Ca) was 1/500 and sera dilution at 1/200.

Detection of Anti-toxoplasma antibodies.

Anti-toxoplasma antibodies in all inoculated groups of mice were detected by IFAT and ELISA. for IFAT, *T. gondii* were diluted with normal saline, spread over microscope slides and kept at -20°C till further use. The slides were fixed in

acetone for 10 minutes, air dried, wahed once in distilled water, dried. The slides were incubated with serial dilutions of sera beginning from 1/2 dilution in a humidified chamber at 37°C for 30 minutes. The slides were washed in PBS three times and once in distilled water. the slides were then dried. Fluorescein conjugated IgG Fraction Goat Anti-mouse Immunolglobulins (IgA & IgG & IgM)(Heavy & light chains specific) (Cappel, Organon teknika Co, PA) (Cat no: ≠ 1211-0231) was added to each well at a dilution of 1/5 and incubated at 37°C in a humidified chamber for 30 minutes. the slides were washed three time in PBS and once in distilled water then air dried. The slides were then mounted with buffered glycerol and examined under a fluorescent microscope.

For detection of anti-toxoplasma antibodies by ELISA, the crude antigen coated plates were incubated with 200µl diluted sera for 2 hours at 37°C, washed with PBS-Tween 20 three times, incubated with 200µl goat antimouse Igs horseradish peroxidase conjugate at 37°C for 3 hours, washed with PBS-Tween 20 three times, incubated with 50µl substrate (0.2 M Na_2HPO_4 + 0.1M citric acid + Ortho-phenylenediamie + H_2O_2) at room temperature for 20 minutes, stopped with 100 µl 2.5 M H_2SO_4 and read at 492 nm with MRX ELISA reader (Dynatech Laboratiories).

Challenging of immunized mice:

The efficiency fo protection of the vaccine was evaluated by Challenging of immunized mice with *T. gondii* Rh strain. the parasites were harvested using the method mentioned previously. The percentage of viable parasites was determined by trypan blue exclusion test to be 80%. a total of 5 x 10^4 live parasites were injected intraperitoneally into the mice. The mice were observed daily for signs of toxoplasmosis and their date of death were recorded.

RESULTS

In testing ant-troxoplasma antiodies in immunized mice, the results presented in table (1) showed that antibodies titers in sera obtained two week post first dose of toxoplasma DNA vaccine were very low as detected by both ELISA and IFAT. Some immunoglobulin (IgG) were detected in sera obtained two week following the booster dose of vaccine. All the mice vaccinated with pGFP-N3 were negative for toxoplasma antibodies (1st group). The second group of mice which vaccinated with pGFP-N3 with p30 insert showed 2 positive case (1/16, 1/16) by IFAT rather than by ELISA. Concerning the third group of mice that vaccinated with pGFP-N3 complexed with cationic liposomes, one mice gave a titer less than 1/2. When pGFP-N3 with p30 insert was complexed with liposomes (fourth gorup), 2 mice

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Table (1): IFAT and ELISA for detection of anti-toxoplasma antibodies after 2 doses of I.M. Toxoplasma DNA vaccine and number of days of survival after challenge with viable Rh strain.

Group	Mouse Number	IFAT	IFAT	ELISA	ELISA	days of survival
		1st serum	2nd serum	1st serum	2nd serum	
1-P GFP-N3	Mouse 1	-ve	-ve	0.1201	0.2405	4
	Mouse 2	-ve	-ve	0.1931	0.2835	6
	Mouse 3	-ve	-ve	0.1460	0.1970	6
	Mouse 4	-ve	-ve	0.5160	0.7170	4
	Mouse 5	-ve	-ve	0.1141	0.2280	6
2-P GFP-N3-p30	Mouse 1	-ve	-ve	0.1151	0.2165	5
	Mouse 2	1/4	1/16	0.1120	0.2910	7
	Mouse 3	-ve	-ve	0.1300	0.2905	7
	Mouse 4	-ve	-ve	0.2012	0.5055	9
	Mouse 5	1/8	1/16	0.2231	0.6240	8
3-.P GFP-N3 + Liposomes	Mouse 1	-ve	>1/2	0.0280	0.1290	5
	Mouse 2	-ve	-ve	0.1170	0.2390	5
	Mouse 3	-ve	-ve	0.2120	0.2230	6
	Mouse 4	-ve	-ve	0.1110	0.2660	6
I-P GFP-N3-p30 + Liposomes	Mouse 1	-ve	-ve	0.1125	0.2115	5
	Mouse 2	1/8	1/16	0.1400	0.2405	5
	Mouse 3	1/8	1/8	0.3001	0.3470	8
	Mouse 4	-ve	-ve	0.2351	0.4365	10
5- Crude Antigen	Mouse 1	1/64	1/128	0.920	1.4512	9
	Mouse 2	1/8	1/16	0.901	1.4804	9
	Mouse 3	1/32	1/128	1.009	1.4951	9
	Mouse 4	1/32	1/128	1.121	1.5963	8
	Mouse 5	1/16	1/128	0.771	1.2582	8

N.B. ELISA O.D. of positive control = 1.3135
negative control= 0.2625, PBS =0.001

5 mice immunized with 2 doses of PBS and challenged with *T. gondii* all died after day 3.

5 unimmuized mice challenged with *T. gondii* died after day 3 and 4.

was positive by IFAT but not by ELISA. the crude toxoplasma antigen vaccine gave positive results by IFAT and ELISA. On challenging all the mice groups with virulent Rh strain of *T. gondii* using a dose of 5 X 10^4, all control gorups were died on day 3 while the vaccinated one showed variation in days of survival (table 1).

DISCUSSION

In the system to test the toxoplasma DNA vaccine in the Balb/C mice, antitoxoplasma antibodies were detected by IFAT in some of animals injected with toxoplasma DNA vaccine but not in the control. There did not appear to be any consistent pattern of the antitoxoplasma antibodies detected by ELISA in the specimens.

P_{30} is a highly imunogenic membrane surface protein of *T. gondii* which is known to induce humoral and cell mediated immunity. Being a surface protein, the antibodies to it could be best detected by IFAT rather than ELISA.

The I/M injection of the vaccine has led to the expression of P_{30} protein is sufficient quantity to produce some immune response. This is important because P_{30} is known to be a difficult protein to express

P_{30} is a highly conformational antigen, whose recognition by antibodies in dependent upon correct disulfide bonding. Bacterial recombinant protein of P_{30} is usually misfolded resulting in the inability of antibodies against native P_{30} to recognize it. Successful conformationall appropriate expression of P_{30} from a MboII-AvaI fragment consisting of 1 960 bp coding sequence and a 310 bp 3' unsaturated region at expression in eukaryotic cell line, the CHO cells (Kim et al., 1994). The insert used by us was a Bgl II-P st fragment which encodes a hydrophobic regions at the 5 'end. The insert also has two ATG start condons.

The lack of antibody response in some of mice immunized with *T. gondii* DNA vaccine may be due to variability in immune response or problems with technique of I.M. immunization.

There appear to be any difference in the IFAT antibody response between the mice immunized with naked DNA vaccine and those immunized with DNA vaccine carried on cationic liposomes. It is possible that any difference was too small to be noted in the study. Moreover, the number of animals tested was small. During the procedure to attach the DNA to liposomes, the DNA in PBS buffere was quantitated and then subjected to ethanol precipitation, the DNA was then reconstituted with HEPES buffered saline and mixed with liposomes. It is possible that some DNA was lost during the ethanol precipitation and hence the amount of DNA used to immunized the mice could have been less.

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All mice were challenged with 5 X 10^4 live *T. gondii* (Rh strain). All control group died while the mice immunized with *T. gondii* DNA vaccine appeared to survive longer, there did not appear strong relationship between the antibody titer and days of survival. It is possible that the challenge dose is too high. Darcy et al., (1992) used 5 x 10^4 live parasites of the Rh strain while Bulow and Boothroyd (1991) used 10^5 live parsities of the C strain. The C strain is less virulent.

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