Evaluation of murine immune responses elicited by a plasmid encoding the *Toxoplasma gondii* SAG1 gene delivered by various routes

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Abstract

The efficacy of four methods of delivering recombinant plasmid DNA into a Balb/C mouse model was evaluated. A recombinant plasmid vector, pCMV-wtp30 was developed which allowed for expression of p30 antigen in mammalian cells. Delivery of this plasmid by intramuscular injection of unconjugated DNA, topical application of unconjugated DNA, topical application of liposomal complexed DNA and intra-peritoneal injection of liposomal complexed DNA were evaluated for their ability to stimulate the cellular and humoral immune responses of their subjects. ELISA studies suggest that none of the methods successfully induced a strong, sustained antibody response against the p30 antigen. Lymphocyte proliferation assays were performed to measure the cellular response. The results indicate that topical application of unconjugated DNA produced the strongest and most consistent cellular response while delivery of liposomal complexed DNA by either route could not efficiently raise cellular responses. Challenge studies, in which the immunised mice were inoculated with 100 live *Toxoplasma* tachyzoites, demonstrated that immunisation with the unconjugated plasmid via the topical route could confer partial protection even in the absence of an antibody response.

Key words: DNA immunisation, *Toxoplasma gondii* p30 antigen, delivery routes

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that infects humans and animals. It is a lethal opportunistic infection in immunocompromised patients e.g. HIV infected or transplant patients on immunosuppressive anti-rejection drugs. SAG1 (also called p30) is the major surface antigen on the parasite membrane and is capable of stimulating strong humoral and cellular responses. Although commercial *T. gondii* vaccines are available (Buxton & Innes, 1995), they do not provide complete, long-lived protection, mainly because they do not stimulate the cellular immunity of the host effectively (Alexander *et al.*, 1996). This is crucial for the development of protective immunity against intracellular protozoan parasites (Alexander *et al.*, 1997).

DNA vaccines have been shown to preferentially stimulate cellular immunity. Studies by Nielson *et al.* (1999) described complete protection after intramuscular (i.m.) immunisation with a plasmid bearing the SAG1 gene. Other reports suggest that the route of delivery, as well as the formulation of the nucleic acid vector (unconjugated or conjugated to carrier molecules) can influence the type of immune response raised (Bohm *et al.*, 1998; Boyer *et al.*, 1998; Macker *et al.*, 1998).

Here, we have constructed an eukaryotic expression vector bearing the SAG1 gene and evaluated the immune response generated by the construct after delivery into murine subjects by several routes. Topical application and i.m. injection of unconjugated DNA plasmid and liposomal delivery of DNA by topical application were selected as they have been demonstrated to successfully transfect DNA *in vivo* (Leitner *et al.*, 2000). Liposomal delivery of DNA by the intraperitoneal (i.p.) route was attempted because it was also the route of inoculation in challenge studies. We wanted to investigate if an immunisation route localised at the site of entry of *T. gondii* would result in better protection. DNA was conjugated to FuGENE™6 in some routes to boost transfection levels while providing adjuvant-like activity (Walker *et al.*, 1992).

Materials and Methods

Construction of recombinant plasmid delivery vector pCMV-wtp30

The RH strain of *T. gondii* is maintained at the Institute for Medical Research through passage in mice. The full length p30 (SAG1) gene (from nucleotides 310-1321) was cloned by PCR from *T. gondii* RH strain genomic DNA using

primers designed based on the published sequence of the SAG1 gene (Burg *et al.*, 1988; Genebank Accession no: M23658); forward (5'AACAGAAGATCTATGTCG GTTTCGCTGCACCACTTCA 3') and reverse (5' AGATCTTCACGCGACACAAGCTGCGATAG 3'). PCR amplification was performed on a thermal cycler (Perkin Elmer) with an initial denaturation at 95°C for 5 mins. 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. and a final extension of 4 mins at 72°C. The cloned product consisted of the entire coding region as well as two putative translation start sites at nucleotides 310 and 362 respectively.

Cloning into the eukaryotic expression vector

Cloning into the 5.5.kb pcDNA3.1/V5/His-TOPO (Invitrogen) was performed using the topoisomerase cloning method. Ligation was performed according to the manufacturer's instructions. 50-100µl of the ligated product were spread on a prewarmed LB agar plate containing 50µg/ml ampicillin selection antibiotic. The plate was incubated overnight at 37°C. Positive colonies were picked and analysed by PCR using the T7 forward primer (5' TAATACGACTCACTATAGGG 3') and the p30 reverse primer followed by digestion with the restriction enzymes *Pst I* and *Hin dIII* to select for the plasmid with the p30 gene inserted in the correct orientation.

Sequencing of the positive clones

Plasmid DNA of three positive colonies was purified by minipreps using modified alkaline lysis and phenol-chloroform-isoamyl alcohol extraction. The p30 gene on the plasmid was sequenced on the ABI Prism™ 310 Genetic Analyzer (Perkin Elmer). Cycle sequencing reactions with

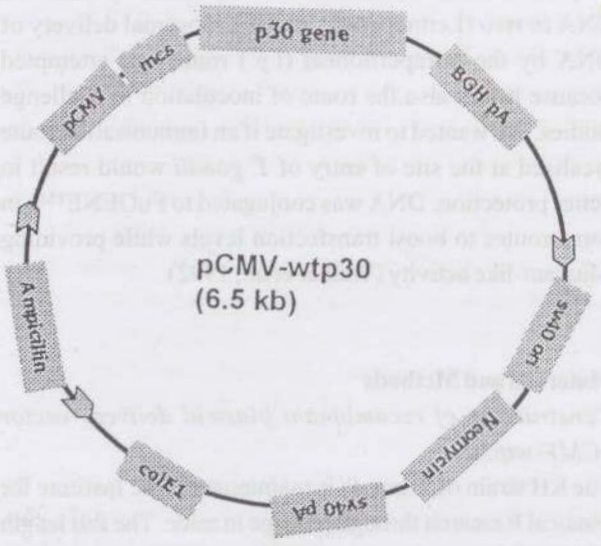

Figure 1. Map of recombinant plasmid pCMV-wtp30

Big DYE terminators were conducted using the T7 forward, p30 forward, an internal antisense primer (5' CTGATTGTTGTCTTGAGGA 3') that bound to nucleotides 677 to 696 and the pcDNA3.1/BGH reverse sequencing primer (Invitrogen) to allow the entire length of the p30 gene to be sequenced. This was repeated in three independent experiments. The sequence data was compared with the published sequence (Genebank Accession no: M23658) using DNASIS version 3.2 (Hitachi Software) and Sequence Navigator (Perkin Elmer) software. One clone was selected and renamed pCMV-wtp30 (6.5 kb) for use as the recombinant plasmid delivery vector (Fig. 1). A reporter plasmid, pCMV-βgal (Invitrogen) expressing the *lac z* gene was used as the negative control.

Large-scale purification of DNA delivery vector

All plasmid DNA used for this study was purified using the Nucleobond Gigaprep kit (Clontech). Plasmids pCMV-wtp30 and the reporter plasmid pCMV-βgal were purified in batches of 500ml LB culture broth derived from a single colony. After purification, isolated plasmid DNA was passed through an endotoxin removal column (Pierce) to remove residual endotoxin. Eluted plasmid DNA was resuspended in normal saline and assayed by UV spectrometric analysis at 280 nm and 260 nm to determine the 260 nm/280 nm purity ratio. Endotoxin levels were measured using the limulous amebocyte lysis (LAL) assay method (Pyrotell, Associates of Cape Cod Ltd), which was sensitive for more than 0.25 EU/ml of endotoxin.

In vitro expression of the recombinant p30 protein

To verify if the recombinant pCMV-wtp30 will correctly express p30 protein, the plasmid was transfected into Vero cells by liposomal mediated transfection. Vero cells were seeded in 25 cm^2 tissue culture flasks (Nunc) at 4.2 x 10^5 cells in 5 ml of DMEM media (Gibco) with 10% FCS growth media. The next day, 6 µl of FuGENE™ 6 were diluted in 100 µl of serum-free DMEM and incubated for 5 min at room temperature. Diluted FuGENE™ 6 was added dropwise to the corresponding tube containing 3 µg of plasmid DNA. The mixture was incubated for 15 min at room temperature. After incubation, the mixture was added dropwise to the cells in growth media. Culture media was swirled gently to distribute the complexed DNA evenly over the cell sheet. Cells were incubated at 37°C in 5% CO_2 for 72 hours before they were assayed for recombinant protein expression.

Detection of p30 protein expression by western blotting

Growth media from the transfected cell sheet was removed and the cells washed three times with sterile PBS. The cell sheet was scraped from the bottom of the flask and

collected into an eppendorf tube. Cells were pelleted by centrifugation and the supernatant removed. The cell pellet was resuspended in 20 µl of Laemmli SDS sample buffer (without β-mercaptoethanol) (Biorad). Suspension was heated at 100°C for 5 min before was 15 µl was loaded onto a 12.5% SDS-polyacrylamide gel together with *Toxoplasma* crude antigen and non-transfected Vero cells and 10 µl of low molecular weight protein markers (Biorad). The gel was electrophoresed at a constant voltage of 110 V and stopped when the bromophenol blue dye front had reached the bottom of the gel. Separated proteins were transferred to a nitrocellulose membrane by electroblotting at 100 V for 60 minutes. The membrane was removed from the transfer buffer and blocked by incubation in blocking buffer PBS-0.5% Tween 20-1% BSA (PBST-1% BSA) for 1 hour with gentle rocking. It was probed with anti-p30 monoclonal antibody TP3 (Research Diagnostics Inc) at a concentration of 10 µg/ml in blocking buffer overnight at 4°C. The next day, the membrane was washed three times in PBST, each time for 10 min with shaking. Anti-mouse IgG-HRP conjugate (ICN) at 1/500 dilution in blocking buffer was applied to the membrane followed by incubation for 1 hour at room temperature. After washing as before, the membrane was washed the final time in PBS for 5 min with shaking. It was subsequently immersed in the substrate solution 4CN (4-chloro-1-napthol) (Sigma) and incubated with shaking until purple precipitate bands were formed or background colour development was observed (up to 45 min).

Intramuscular (i.m.) immunisation

4 week-old BalbC mice were anaesthetised by diethylether inhalation and 100µg of pCMV-wtp30 (1µg/µl in saline) were injected into each hind leg muscle, as described in Lee *et al* (1999). Injection was performed three times at three-week intervals. Tail bleeds were collected on Days 0, 5, 14, 30, 49 and 77.

Topical immunisation

Immunisation was performed as described in Fan *et al.* (1999). Immunisation was performed three times at three-week intervals. Tail bleeds were collected on Days 0, 5, 14, 29 and 77.

Topical immunisation of liposomal complexed DNA

DNA liposome complexes were prepared. FuGENE™ 6 was first concentrated by evaporation to reduce the total volume of diluent required to <250µl. FuGENE™ 6 (60µl before concentration) was diluted by addition into 113µl sterile HEPES buffered saline buffered and incubated for 5 min at room temperature. 30µg pCMV-wtp30 were added to the diluted FuGENE™ 6 and incubated for 15-30 min to allow the formation of DNA:FuGENE™ 6 complexes. Complexes were applied as for topical immunisation above. Immunisation was performed three times at three-week intervals. Tail bleeds were collected on Days 0, 5, 57 and 79.

Intraperitoneal (i.p.) immunisation of complexed DNA

DNA liposome complexes were prepared according to the manufacturer's instructions. 60µl of FuGENE™ 6 were diluted by addition into 940.8µl sterile saline buffered with HEPES and incubated for 5 min at room temperature. 20µg DNA were added to the diluted FuGENE™ 6 and incubated for 15-30 min to allow for formation of DNA:FuGENE™6 complexes, which were subsequently injected i.p. into mice. Immunisation was performed three times at three-week intervals. Tail bleeds were collected on Days 0, 5, 14, 20, 42 and 77.

Assay of humoral anti-p30 antibody responses by ELISA

ELISA wells (Immulon) were coated with 100 µl recombinant p30 (rp30) (Roche) in carbonate buffer (5 µg/ml) and incubated overnight at 4 °C. The next day, wells were blocked with 250 µl blocking buffer, PBS Tween 20-BSA (1%) for 1 hour at room temperature (RT). Wells were washed with PBS Tween 20 for three times at 5 min per wash. Serum from tail bleeds, diluted 1/50 in blocking buffer, was dispensed at 100 µl/well in triplicate and incubated shaking for 2 hours at 37°C. (Note: in all ELISAs serum from naïve mice served as negative controls while the positive control was serum from rp30 immunised mice. Blanks were duplicate wells that contained 100 µl of blocking buffer only). After incubation, sera were discarded and the wells were washed with PBSTw20 as before. Goat anti-mouse IgG+M+A-alkaline phosphatase (AKP) (KPL) conjugated antibody was added at 1/4000 dilution in blocking buffer at 100 µl/well. Wells were incubated for 1 hour with shaking at 37°C. After incubation, the wells were washed with PBSTw20 for three times as above. At the final wash, wash solution was incubated for 10 min in the wells. 100 µl of AKP substrate disodium p-nitrophenyl phosphate (Sigma 104 Phosphatase Substrate tablets) was dispensed and incubated for 1 hour shaking at room temperature. The reaction was stopped with 100 µl 3M NaOH. Results were read on an ELISA reader (Dynatech) by measuring absorbance at 410 nm. Absorbance readings were adjusted to reflect the positive mean value of 1.2 OD.

Table 1. Number of mice for each route that were challenged with 100 live tachyzoites

Route	*Plasmid*	No. of mice challenged
Intramuscular (i.m.) injection	pCMV-wtp30	4
	pCMV-β gal	4
Topical (unconjugated DNA)	pCMV-wtp30	3
	pCMV-β gal	3
Topical (complexed DNA)	pCMV-wtp30	5
	pCMV-β gal	4
Intraperitoneal (i.p.) injection	pCMV-wtp30	4
	pCMV-β gal	4
TCA antigen (positive control)	-	10
Non-immunised	-	10

Isolation of splenocytes

Splenocyte proliferation assays were performed three weeks after the third immunisation. Splenocytes were isolated as described (*Current Protocols in Immunology, pg.3.1.3, 1993*). After the final washing step the cells were resuspended in wash media to a cell concentration of 5×10^6 cells/ml. 100 µl of cell suspension were dispensed into wells of a 96 well round bottomed microtitre plate (Nunc Cat no: 163320). The test antigen, recombinant p30 (rp30) protein was diluted in wash media to concentrations of 1000 ng/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml and 10 ng/ml. 100 µl of each antigen concentration were added in triplicate to the cells. Wells without antigen served as negative controls. Phytohaemagglutinin (PHA) (10 µg/ml, Sigma) was added to triplicate wells as positive control. Plates were incubated in a 37°C CO_2 incubator for 72 hours and observed daily for signs of contamination.

Assay of the proliferative response

After 72 hours, 50 µl of spent culture media was removed from each well and replaced with 50 µl (0.5 µCi/well) of tritiated thymidine (^{3}Th) (Amersham) diluted in sterile 0.85% saline. Cells were pulsed with ^{3}Th for 24 hours and subsequently harvested using a cell harvester (Skatron), processed and counted in a β-counter (Wallac).

Challenge studies

RH strain tachyzoites were isolated from the peritoneal cavity of infected mice. The tachyzoites were washed in sterile normal saline and passed through a 3 µl syringe filter (Nucleopore) to remove peritoneal lymphocytes. Parasite concentration was adjusted to 100 parasites/500 µl in 0.85% saline. At this concentration 50% of naïve mice succumbed by day 7. 500 µl of this suspension were injected into the peritoneal cavity of the mice (Table 1). The positive controls were mice that had been immunised with *Toxoplasma* crude antigen (7 doses of 100 µg/100 µl i.p. at fortnightly intervals). The number of days of survival was recorded.

Results

The entire coding region of the p30 gene was successfully inserted into the pcDNA 3.1/V5/His-TOP eukaryotic expression vector between nucleotides 953 and 954, creating the recombinant plasmid pCMV-wtp30 (Fig. 1).

Large scale plasmid purification and endotoxin removal consistently yielded plasmid with a purity ratio of between 1.7-1.9 and endotoxin levels of less than 0.36 E.U., which meet the recommended limit for injectable substances, which is less than 50 E.U./mg (Butler, 1996).

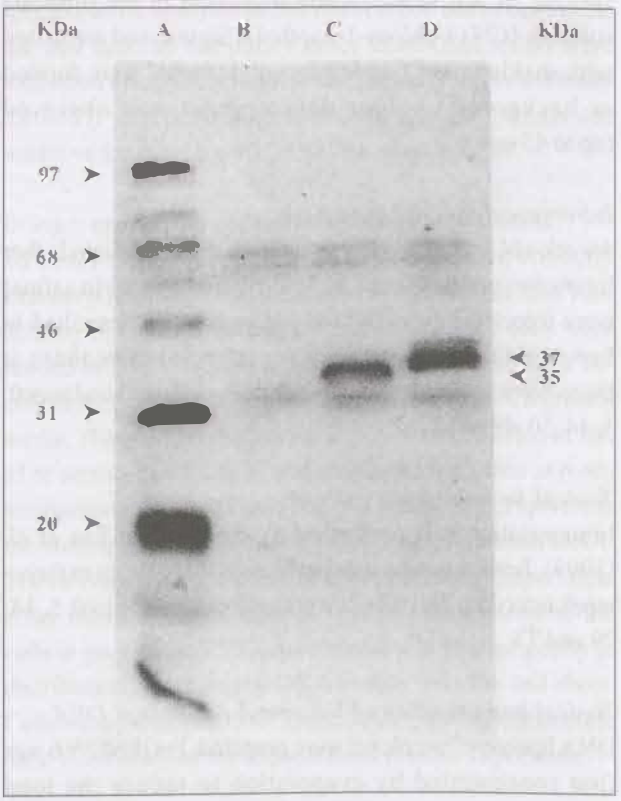

Figure 2. Immunoblot of Vero cells transfected with pCMV-wtp30 after probing with the anti-p30 monoclonal antibody, TP3, followed by detection with HRP conjugated goat anti-mouse IgG antibody and visualisation using substrate 3CN. Lane A: low protein mass marker, lane B: non-transfected Vero cells (negative control) lane C: toxoplasma crude antigen (positive control), lane D: Vero cells transfected with pCMV-wtp30.

In vitro protein expression studies and western blotting using the anti-p30 diagnostic monoclonal antibody TP3 detected the expression of recombinant p30 (Fig. 2), although the protein appeared to be of a heavier molecular weight (~34 kDa) compared to p30 isolated from sonicated *T. gondii* tachyzoite. This may be the result of excessive glycosylation by the mammalian cell line as reported by other groups (Odenthal-Schnittler *et al.*, 1993; Kim *et al.*, 1994; Nielson *et al.*, 1999), although it does not appear to have blocked the epitope of the monoclonal antibody. This demonstrates that the recombinant p30 gene on the plasmid is functional and eukaryotic cells can fold the protein correctly.

ELISAs performed on the test bleeds suggested that the humoral response to p30 in immunised mice was generally low to non-responsive (Fig. 3). Most of the immunised mice, regardless of the method of delivery, recorded absorbance values that were either baseline or less than twice the value of the negative. The highest responses were observed at Day 5 after the first immunisation, where the OD readings increased about twice the mean negative value. In spite of this, by day 77, before cell proliferation or challenge studies, the OD values for all the mice had dropped below the baseline Day 0 OD (Fig. 3). The highest OD recorded, 0.311 for i.m. immunised mouse G1.3, was four times lower compared to the mean positive of 1.2 obtained after three unmunisations with rp30 antigen. ELISA with anti-mouse IgM or IgG was not conducted because of the low values obtained in the anti-mouse Ig G+M+A assay.

The cell proliferation data is presented in the form of stimulation indexes (S.I.), which is the ratio of [mean cpm of sample-blank cpm] / [mean cpm of unstimulated cells-blank cpm]. Here, a S.I. of >1.3 which is the highest S.I. obtained in negative control assays was considered a proliferative response to p30 antigen (Table 2). Lymphocyte proliferation was mainly observed in mice immunised with unconjugated DNA. Mice immunised i.m. both responded to the rp30 antigen, although not in a dose responsive manner. Two of three mice immunised by topical application responded in a dose responsive manner. Immunisation using conjugated DNA saw only one mouse, which had been topically immunised displaying non-dose responsive proliferation. None of the i.p. immunised mice showed specific p30 cellular responses.

In the challenge studies, of the four delivery systems tested, all immunised mice survived marginally longer than the non-immunised mice did. The results are summarised in Table 3. Statistical data of the variance between the three groups (non-immunised, pCMV-β gal immunised and pCMV-wtp30 immunised mice) was calculated using the 1-way ANOVA Test, where a p value of < 0.05 suggests that there is significant difference between mice immunised with pCMV-wtp30 or pCMV-β gal and non-immunised mice. Two mice survived until the study was terminated at Day 30. They were both immunised with the plasmid pCMV-wtp30 that was conjugated with FuGENE™ 6, one via i.p. injection, the other by topical application. None of the mice immunised with the reporter plasmid:FuGENE™ 6 conjugate survived beyond Day 9.

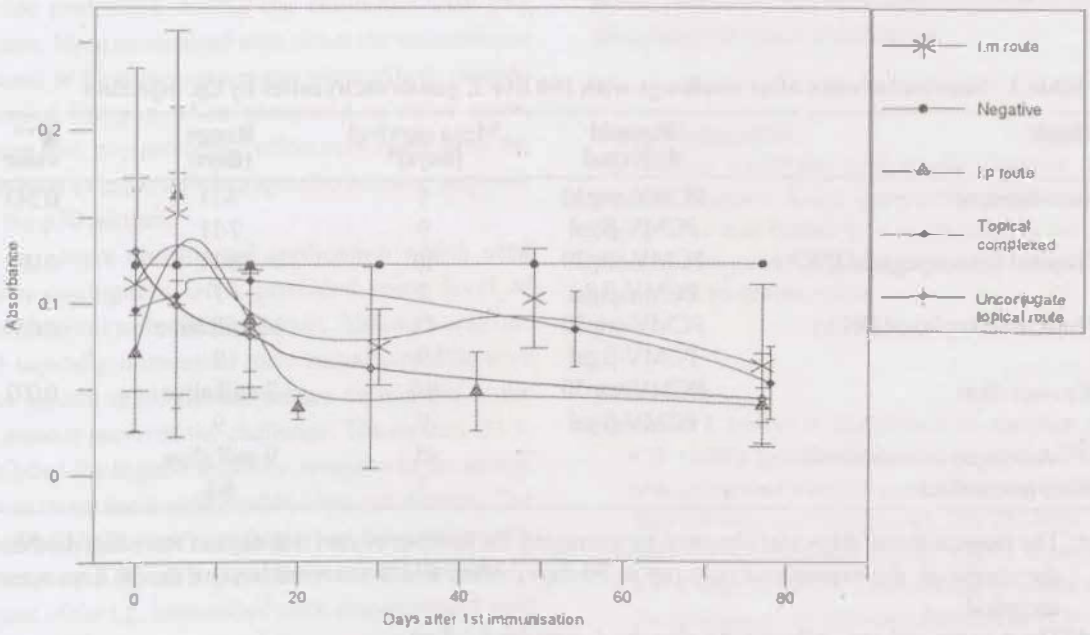

Figure 3. Anti-p30 antibody responses from mice immunised with pCMV-wtp30 by various routes over the course of three immunisations.

Table 2. Stimulation index (S.I.) in response to various concentrations of rp30 antigen (ng/ml)

Mouse	0	10	50	100	500	1000	PHA
Unconjugated pCMV-wtp30							
Intra-muscular injection							
G1.2	1.0	4.8	1.9	2.8	2.3	2.1	21.7
G2.10	1.0	6.5	N/A	6.5	6.2	6.4	4.5
Topical application							
G1.2	1.0	2.9	3.8	4.8	4.5	3.9	20.4
G2.5	1.0	0.7	1.0	1.1	1.1	1.6	3.3
G2.9	1.0	12.0	17.7	11.6	24.5	55.3	28.9
PCMV-wtp30:FuGENE 6 conjugated DNA							
Topical application							
G2.3	1.0	N/A	N/A	1.6	0.8	1.4	N/A
G2.5	1.0	N/A	7.4	1.5	N/A	2.2	7.5
G2.6	1.0	0.1	0.3	0.7	0.6	0.7	5.2
Intraperitoneal injection							
G1.1	1.0	0.9	0.7	0.9	1.0	1.0	2.4
G1.2	1.0	0.7	0.7	1.4	1.7	1.7	3.3
G1.5	1.0	0.3	1.1	N/A	1.1	1.1	1.2
G2.2	1.0	N/A	N/A	0.4	N/A	N/A	0.9
Controls							
TCA immunised positive control							
1	1.0	1.3	1.7	0.5	1.0	1.0	1.8
2	1.0	1.5	1.8	1.1	N/A	N/A	N/A
Non-immunised							
1	1.0	0.8	0.8	0.9	0.9	0.8	3.3
2	1.0	0.8	0.8	0.9	0.6	0.8	1.6
3	1.0	0.7	1.1	0.6	1.2	0.7	2.6
4	1.0	0.8	0.9	1.3	1.7	0.7	5.7
5	1.0	1.0	0.7	0.5	1.1	0.8	3.6

Table 3. Survival of mice after challenge with 100 live *T. gondii* tachyzoites by i.p. injection

Route	Plasmid delivered	Mean survival (days)*	Range (days)	p** value
I.m. injection	PCMV-wtp30	9	7-11	0.243
	PCMV-β gal	9	7-11	
Topical (unconjugated DNA)	PCMV-wtp30	10	8-11	0.008
	PCMV-β gal	7	7	
Topical (complexed DNA)	PCMV-wtp30	11	11-still alive	0.037
	PCMV-β gal	9	9	
I.p. injection	PCMV-wtp30	8.6	7-still alive	0.092
	PCMV-β gal	9	9	
TCA antigen (+ve control)	-	11	9-still alive	
Non-immunised	-	7	6-8	

* The mean survival days was obtained by averaging the number of survival days of mice that died during the course of the experiment only (up to 30 days). Mice which survived beyond the 30 days were not included.

** The p value was was calculated using the 1-way Anova Test.

Discussion

In vitro transfection assays in Vero cells demonstrated that recombinant plasmid pCMV-wtp30 was expressing p30 protein which could be recognised by a diagnostic anti-p30 monoclonal antibody, TP3. However, in *in vivo* studies, humoral assays showed that little antibody response was generated against p30, a normally highly immunogenic antigen. This may be due to the presence of both the reading frames on the gene. The 145 bases after the second translation site is believed to encode a membrane localisation signal that results in attachment of the protein to the membrane by a GPI anchor. Thus, our protein may have been expressed and localised on the membrane of the *in vivo* transfected cell after immunisation, which may have lowered its chances of encountering an antigen presenting cell. Other workers have reported failure to generate antibody responses after DNA delivery (Hosie *et al.*, 1998, Han *et al.*, 1999, Le *et al.*, 2000). It is likely that the methods attempted here were not optimised for the generation of antibody responses.

Topical application of unconjugated pCMV-wtp30 DNA appeared to confer the most protection. It raised specific anti-p30 cellular responses in two of three mice tested while the results of the challenge studies indicated that immunisation with pCMV-wtp30 did confer significant protection (p value <0.008) by prolonging the length of survival after inoculation. The post-hoc (Duncan's) test indicated that immunisation with pCMV-wtp30 significantly increased the length of survival after challenge with *Toxoplasma* tachyzoites. In contrast, i.m. immunisation, while shown to induce specific cellular responses in both mice tested against rp30, was not able to provide protection during the challenge with live tachyzoites. Mice immunised with either the recombinant p30 plasmid or the irrelevant reporter plasmid both enjoyed an extended lifespan when compared to naïve mice, suggesting that any protective effect seen came from the immunisation event, not from a specific immune response against the p30 antigen.

The i.p. route and topical application which used liposomal conjugated DNA provided some level of protection against tachyzoite infection. Although only one out of 3 topically immunised mice raised a proliferative response against rp30, another mouse immunised in the similar manner survived the challenge. The mouse, GT.3, had exhibited the highest antibody response in the group, which was twice the baseline value (data not shown). The p value of 0.032 although significant, was not confirmed in the subsequent Duncan's post-hoc analysis. Similarly, while none of the i.p. immunised mice demonstrated rp30 specific proliferation, one of the mice survived the challenge study. It is possible that the DNA:lipid ratio has not been optimised for *in vivo* delivery. The adjuvant-like activity of the liposome may account for the increase survival rates in the absence of specific cellular responses against rp30.

These findings are in contrast with the reports by Nielson *et al.* (1999), which found 80% protection in Balb/C mice after i.m. immunisation with a p30 DNA vaccine that was constructed by inserting an SAG1 minus the membrane localisation signal into a vector containing a secretory tag.

Both i.m. immunisation and the topical application routes have been known to promote cellular responses (Fan *et al.*, 1999). However, i.m., immunisation normally induces higher levels of proliferation compared to topical application, but this was not observed here. This could be attributed to either processing errors or variability in the injection technique as a result of worker inexperience. Not all of the DNA may have entered the mouse muscle leading to lower responses. One explanation for the high responses through topical application may be that the skin functions as an immune barrier against invading pathogens. Not only is it rich in antigen presenting cells and lymphoid tissue, more importantly, it has the ability to mount immune responses against small amounts of antigen. By transfecting hair follicles, which have been previously identified as a portal for DNA and protein entry into the skin (Li & Hoffman, 1995), low p30 antigen expression in the right location may have resulted in higher immune responses compared to i.m., immunisation. Nonetheless, the cell proliferation and challenge studies have demonstrated that nucleic acid immunisation can confer partial protection against *T. gondii* infections even without stimulation of humoral immunity.

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