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Detection of Progeny Immune Responses after Intravenous Administration of DNA Vaccine to Pregnant Mice

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ABSTRACT

A number of factors influence the development of tolerance, including the nature, concentration and mode of antigen presentation to the immune system, as well as the age of the host. The studies were conducted to determine whether immunizing pregnant mice with liposome-encapsulated DNA vaccines had an effect on the immune status of their offspring. Two different plasmids (encoding antigens from HIV-1 and influenza virus) were administered intravenously to pregnant mice. At 9.5 days post conception with cationic liposomes, injected plasmid was present in the tissues of the fetus, consistent with trans-placental transfer. When the offspring of vaccinated dams were immunized with DNA vaccine, they mounted stronger antigen-specific immune responses than controls and were protected against challenge by homologous influenza virus after vaccination. Moreover, such immune responses were strong in the offspring of mothers injected with DNA plasmid 9.5 days after coitus. These results suggest that DNA vaccinated mothers confer the antigen-specific immunity to their progeny. Here we describe the methods in detail as they relate to our previously published work.

INTRODUCTION

Most vaccines intended for human use are administered to infants and children. Due to the immaturity of their immune system, newborns exposed to foreign antigens are at risk of developing tolerance rather than immunity (2-7). For example, if antigen is administered shortly after birth, forbidden clones can emerge and induce such tolerance (2-7). A number of factors influence the development of tolerance, including the nature, concentration and mode of antigen presentation to the immune system, as well as the age of the host (8,9). Over the past decade, there has been considerable interest in the use of DNA vaccines to prevent infection by pathogenic viruses, bacteria and parasites, with phase I clinical trials being initiated against malaria, HIV-1 and hepatitis B virus.

In the present study, we confirmed that plasmid DNA administered to pregnant mice could reach the fetus through the placenta. This was true both of DNA vaccines encoding the *env* gene of HIV-1 as well as those encoding the influenza virus matrix (M) and nucleoprotein (NP) genes. Analysis of the immune response of offspring whose mothers were immunized with the influenza DNA vaccine indicate that these progenies had enhanced level of protection against the same virus infection.

MATERIALS AND METHODS

Animals

We used 6-10-week old BALB/c female mice purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were allowed free access to sterile food and water.

Viral protein expression plasmids and antibodies

A pME18S-M expression plasmid was constructed with the pME18S expression vector into which M region cDNA from influenza virus strain A/PR/8/34 (H1N1) had been inserted (13). pME18S empty vector was used as a control plasmid for A/PR/8/34 challenge. The expression of the proteins was confirmed by Western blot analysis (10). pCMV160IIIB encoding the *env* gene of HIV-1 strain IIIB has been described in detail in our previous report (11). DNA vaccines of NP (A/pCMV-V1NP) and HA (V1J-HA (PR8)) genes of the A/PR/8/34 strain (14-16) were the kind gifts from Drs. J. J. Donelly and D. Montgomery, Merck Research Lab., West Point, PA. To confirm that plasmid DNA was transferred through the placenta, a *lac*Z expression plasmid containing a chicken β-actin promoter was also used.

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Virus Preparation (Protocol I)

Mouse-adapted influenza A/PR/8/34 (H1N1) viruses were used in this study. Maudin-Darby canine kidney (MDCK) cells were cultured with Eagle's minimum essential medium (MEM, Nissui Corp. Tokyo, Japan) containing 10% FCS at 5% CO_2, 37°C. Viruses were harvested from infected MDCK cells and titrated according to the plaque formation method.

DNA immunization (Protocol II)

Mothers were injected intravenously (i.v.) with DNA vaccine before or after coitus. Preparations containing various doses of the DNA vaccine were encapsulated into liposomes (12).

Briefly, a 1:1 volume of 6.01 mg/ml 3β[N-(N'N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and 5.99 mg/ml dioleoylphosphatidylethanolamine (DOPE) in chloroform was mixed with an evaporator set at 40°C for 1 hr. Five volumes of 20 mM HEPES buffer (pH 7.8) was added into the mixture, mixed and kept at 4°C overnight to completely dissolve the pellet. Then, the resultant solution was sonicated with a Sonifier 250 (Branson, Danbury, CT) set at output control 2-3; duty cycle 30, for 5 min. The cationic liposomes were stocked at 4°C and used within 2-3 months. Prior to administration, an appropriate amount of DNA in phosphate-buffered saline (PBS), pH 7.2, was mixed with the liposome solution at a volume ratio of 1:1. The pregnant mice were i.v. administered the DNA vaccine or empty vector on various days after post conception (p.c.) to assess immunogenicity. Six weeks after birth, their offspring were injected intramuscularly (i.m.) in gastrocnemius muscles with 20-50 μg of the same expression plasmid or the empty vector. For the influenza virus challenge experiment 50 μg each of plasmids expressing influenza M and NP protein was administered into pregnant mice by i.v. route. The same amount of DNA plasmids was administered to offspring by i.m. route.

X-gal staining

Mouse fetus was cut in two and washed with PBS once followed by incubation at 37°C for 30 min with X-gal staining buffer (5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, 20 mM $MgCl_2$, 1 mg/ml X-gal).

FISH analysis (Protocol III)

FISH analysis was performed essentially by the method of El-Naggar *et al*. (17). A 564-bp HIV *env* region fragment (nt 1569-2133) was amplified from pCMV160IIIB plasmid (10) using primers (5-ATGTGTAACACCTCAGTCATTAC and TTATCTTTTTTCTCTCTGCACCAC-3). The PCR product was purified from 1.5% agarose gel using QIAquick gel extraction kit (Qiagen) and was labeled with digoxigenin-11-dUTP by nick translation. The labeled product (300-560 bp DNA) was confirmed on 2% agarose gel and used for a hybridization probe. The tissue samples were taken from mice to which 50 μg of HIV plasmid pCMV160IIIB (11) or influenza plasmid V1J-HA(PR8) (14) with liposomes were administered and sliced to prepare histological examination. The samples were denatured and hybridized with digoxigenin-labeled probes using a previously described method (17). After hybridization, the slides were washed and stained with an anti-digoxigenin rhodamine (a red fluorochrome: Boehringer Mannheim, Germany). The slides were counterstained with 4,6-diamino-2-phenylindole dihydrochloride (DAPI). Images were made with a Nikon SA fluorescence microscope (Nikon Corp., Tokyo, Japan) and a Charge Coupled Device (CCD) camera interfaced with a Cyto Vision (Applied Imaging, Sunderland, UK).

Cytokine ELIspot assay (Protocol IV)

The cytokine ELIspot assay was performed with minor modifications, as previously described (18,19). Briefly, 96-well microplates (MAIPS4510, Millipore, Bedford, MA) were coated with anti-mouse IFN-γ rat mAb (PharMingen), and after adding cells isolated from the spleen 7 days after immunization, plates were incubated in a 5% CO_2 atmosphere at 37°C with or without 10 μg/ml of V3 peptide. After a 24-hour culture, plates were washed and incubated again for 2 hours with biotinylated anti-mouse IFN-γ mAb (PharMingen). Then, after staining with alkaline phosphatase, the spots in each well were counted using a computer assisted video image analysis system (Zeiss Co., Germany). By applying the proper dilution factor the total number of cytokine-secreting cells was calculated.

Virus challenge

Under light diethyl ether anesthesia, the offspring were simultaneously infected with virus at day 10 after immunization with the same plasmid DNA as that administered to their mothers. Five lethal doses (LD_{50}) of influenza A/PR/8/34 (H1N1) in 30 μl of PBS were administered by the intratracheal route using a 24-gauge stainless steel animal feeding tube (Popper & Sons, New York, NY). The mortality rate was determined after 20 days.

Statistical analysis

Statistical analysis for comparison of two groups was conducted using an unpaired *t*-test or one-way factorial analysis of variance (ANOVA) for distribution parameters. Significance was defined as $p<0.05$ in both analyses.

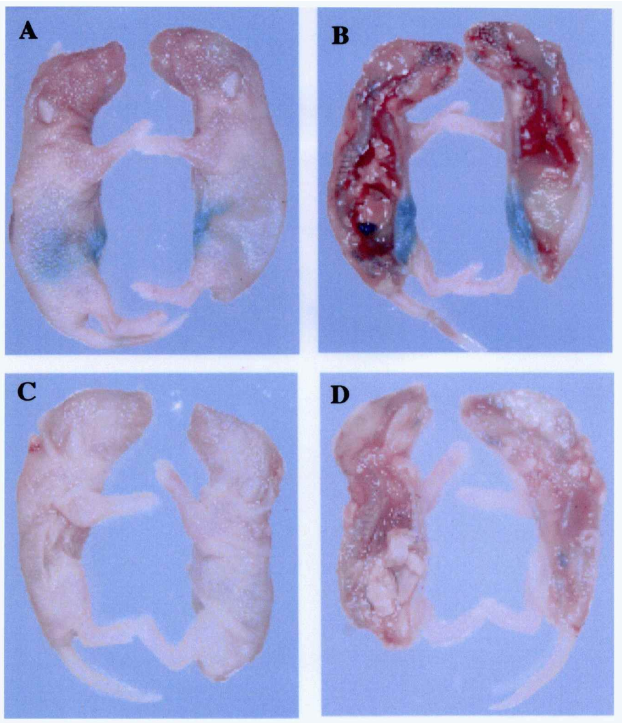

Fig. 1: Gene expression of *lacZ* in mouse fetus. A and B, Stained tissue of a fetus cut in two, whose mother received 30 µg of *lacZ* plasmid with liposomes at day 9.5 postcoitus (p.c.); C and D, stained tissue of a fetus whose mother received 30 µg of empty plasmid with liposomes at day 9.5 p.c.

RESULTS

Gene transfer into fetuses

Initial studies examined whether DNA plasmids could be transmitted through the placenta of pregnant mice. To evaluate plasmid uptake and expression in fetal tissue, a plasmid expressing the *lacZ* gene was utilized. To increase the uptake of this plasmid, it was liposome-encapsulated prior to *i.v.* delivery (8). Tissues from newborn mice from mothers injected with the *lacZ* plasmid and liposomes 9.5 days p.c. showed strong expression of that gene (Fig. 1). Of particular interest was the intense staining in the umbilical region of the fetuses. Examination by the FISH method confirmed that abundant plasmid DNA had been transmitted to the fetuses (Fig. 2). We found abundant plasmid DNA in spleen, liver, lung, and other tissues (data not shown).

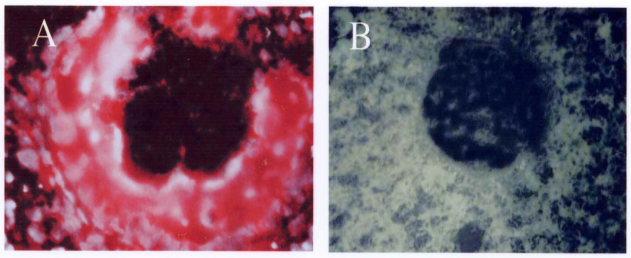

Fig. 2: FISH analysis. Vertebra of a fetus whose mother received HIV plasmid pCMV160IIIB (A) or influenza plasmid V1J-HA (PR8) (B) with liposomes at day 9.5 p.c. These samples were reacted with HIV *env* region fragment labeled with digoxigenin-11-dUTP, followed by staining with an antidigoxigenin rhodamine. Red fluorochrome indicates the presence of HIV-IIIB DNA.

ELIspot analysis using spleen cells from immunized offspring of vaccinated mothers was performed (Table 1). When stimulated *in vitro* with vaccine-encoded antigen, a significant increase in the number of spleen cells secreting IFN-γ was observed.

Table 1: ELIspot analysis of IFN-γ producing spleen cells from DNA-vaccinated mice whose mothers had been injected with the same vaccine during pregnancy.

	Immunogen administered to		IFN-γ producing cells
	Pregnant mothers	Progenies	(Spot/10^6spleen cells)
1.	pCMV160IIIB	[illegible]160IIIB	42.6±9.6*
2.	pCMV160IIIB	mmune	20.6±6.7
3.	Empty vector	160IIIB	28.2±3.5*
4.	Empty vector	Empty vector	16.7±6.3
5.	Non-immune	Non-immune	13.9±5.9

† (bracket between rows 1 and 3)

At day 9.5 p.c., pregnant BALB/c mice were i.v. injected with 50 µg of pCMV160IIIB or empty vector with liposomes. Six weeks after birth the offspring were immunized i.m. with 50 µg of the same plasmid or empty vector as received by their mothers. After 7 days, spleen cells were collected and cocultured with V3 peptide for 24 hours. Data represent means±SE of 6-8 mice. *Indicates significant difference ($p<0.05$) compared to the empty vector (control). †Means a significant difference between the two indicated values. Data from two other experiments showed similar results.

Challenge test with influenza virus

To examine the immunoprotective effect of maternal vaccination with a DNA vaccine against influenza virus, offspring were immunized with 50 µg of the same vaccine at 6 weeks of age. Seven days later they were challenged with influenza virus A/PR/8/34. More than 70% of the offspring of vaccinated mothers survived (Fig. 3), whereas only 20% of the offspring of non-vaccinated mothers survived. All of the non-immunized offspring whose mothers received liposomes alone or were not immunized had died.

The timing of maternal DNA vaccination on the capacity of offspring to develop protective immunity was then examined. Whereas <20% of normal vaccinated mice (and offspring of mothers vaccinated 20 days prior to mating) survived the

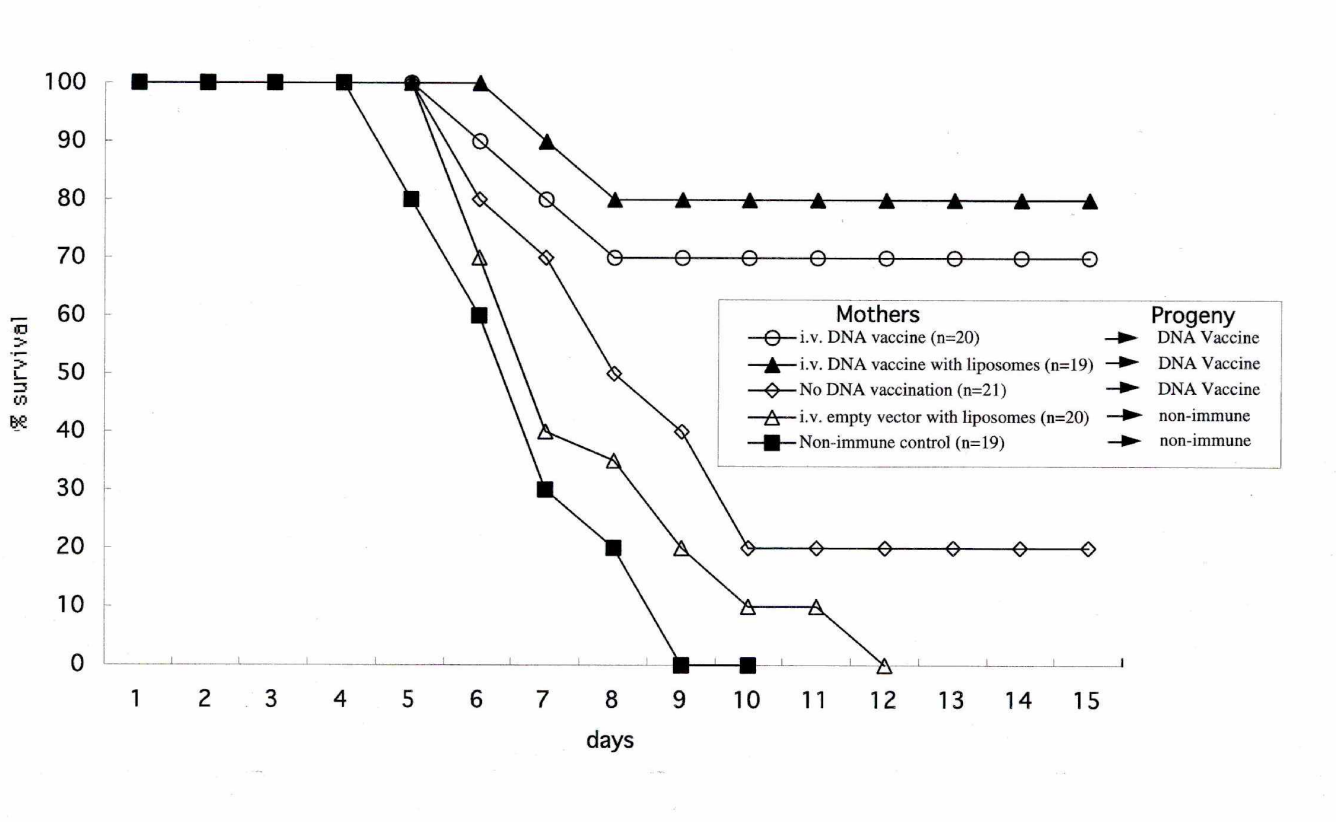

Fig. 3: Protection of offspring against a lethal A/PR/8/34 influenza virus challenge. Day 9.5 p.c. pregnant BALB/c mice were i.v. injected with 50 μg each of pME18S-M and pCMV-V1NP with or without liposomes. Six weeks after birth, their offspring were immunized i.m. with a total of 50 μg of the same plasmid DNA with liposomes. In one group, offspring of mothers that had received DNA vaccine with liposomes were not administered vaccine. In another group, the mother and progenies received only empty vector. Non-immunized normal mice were used as the other control. After 7 days, all mice were challenged with 5xLD50 of A/PR/8/34 virus. The percentage of survival of these mice was studied for another 15 days. *n*, number of mice.

challenge, >50% of the offspring of immunized mothers survived (Table 2).

These findings indicate that immunization of mothers with a DNA vaccine against the influenza virus improves the ability of their offspring to develop protective immunity against viral challenge post vaccination.

DISCUSSION

Two independent techniques were used to establish that plasmid DNA administered to pregnant mice could reach the fetus. One is the administration of a β-gal encoding plasmid which allowed for the direct identification of protein expression in neonates. Intense staining of the placenta was consistent with transplacental migration of the plasmid. The other technique is the FISH method which was used to directly detect plasmid DNA in fetal tissue (Fig. 2). Our results confirm and extend the previous finding (10) that in mice a β-gal plasmid can be transmitted through the placenta to the fetus. Of particular importance, we established that such trans-placental transfer influences the recipients' subsequent capacity to mount an immune response against the plasmid-encoded antigen. This was manifested by improved cellular immunity (Table 1) and higher levels of pathogen-specific protection (Fig. 3 and Table 2).

These studies were also performed to test the hypothesis that the administration of a DNA vaccine during pregnancy may induce antigen-specific tolerance in the offspring, as suggested by the clonal selection theory of Burnet (3,4,20). Although pregnant mice were immunized with various doses of several different DNA vaccines, we found that immunity but not tolerance was elicited in the fetus (Fig. 3 and Tables 1 & 2). Using this technique, we did not observe antigen-specific immune tolerance in progeny as reported by Mor *et al*. (21). This could reflect our use of a different plasmid (Mor *et al*. detected tolerance following neonatal immunization with a plasmid encoding the circumsporozoite protein of malaria), or the very limited amount of plasmid actually transferred trans-placentally. Indeed, Ichino *et al*. demonstrated that neonatal tolerance was dose-dependent, and could be reliably induced

only when >10 μg of plasmid was injected into newborn mice (8). This is consistent with other reports showing that low dose antigen can induce immune responsiveness, while high dose immunization can induce tolerance in young recipients (9,22,23). This might be in support of Burnet's theory that high levels of neonatal antigen can trigger clonal deletion (4,8).

Table 2: The importance of timing the DNA immunization of pregnant mothers against A/PR/8/34 virus challenge.

DNA vaccination of		Survival after immunization
Pregnant mothers	Progeny	Survivors/total (%)
1. DNA vaccination		
20 days before coitus	DNA vaccination	3/19 (15.8)
day 5.5 p.c.	DNA vaccination	5/20 (25.0)
day 9.5 p.c.	DNA vaccination	11/21 (52.3)*
day 14.5 p.c.	DNA vaccination	12/19 (63.2)*
day 9.5 p.c.	no vaccination	3/18 (16.7)*
2. Empty vector		
day 9.5 p.c.	DNA vaccination	2/22 (9.1)
3. Non-immune control	Non-immune	1/19 (5.3)

Pregnant BALB/c mice were i.v. injected with 25 μg each of A/pCMV-V1NP$^+$pME18S-M and liposomes on day 5.5, 9.5 or 14.5 p.c. Six weeks after birth, all offspring were immunized i.m. with 50 μg of the same plasmid DNA as received by their mothers. After 7 days, these mice were challenged with 5xLD_{50} of A/PR/8/34 and the per cent survival was determined after another 20 days. Data represent means±SE of 19-22 mice. *Indicates statistically significant difference ($p<0.05$) between non-immune control group. Data from another separate experiment showed similar results.

When offspring of vaccinated mothers were immunized at 6 weeks of age with the same DNA vaccine, they displayed significant anamnestic responses. Re-immunization was required, however, since trans-placental transport of plasmid alone did not trigger strong immune responses in the newborn, or provide adequate protection from infection (Fig. 3 and Table 2). On the other hand, re-exposure of these mice to vaccine at 6 weeks of age elicited a strong, protective immune response characterized by antigen-specific antibody, CTL and cytokine responses. The administration of DNA vaccine into amniotic fluid induced a high level of protective immunity (24). In the present study, when DNA vaccine was given to mothers, Ag-specific acquired immunity was induced in their offspring. Therefore, this method may be effective in the prevention of pertussis, hepatitis type B and C, mumps, rubella and various other infections occurring in infants as well as animals.

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PROTOCOL I: PREPARATION OF INFLUENZA VIRUS

1. Culture MDCK cells (ATCC, No. CCL-34) in a 10 cm^2 dish with Minimum Essential Medium (MEM) containing 10% FCS and antibiotics in a CO_2 incubator at 37°C.
2. Aspirate medium from a confluent cell monolayer.
3. Wash cells once with PBS.
4. Remove PBS and infect cells with 1 ml of FCS-free MEM containing 10^4 pfu of influenza virus.
5. Place in a CO_2 incubator at 37°C for 1 hr, rocking the dish at 15-30 min intervals to keep cells moist.
6. After 1 hr of incubation, add 10 ml of MEM medium containing 10% BSA and antibiotics, and place in a CO_2 incubator at 37°C for 3 days.
7. Store supernatant at –80°C until use.

Titration of influenza virus

1. Prepare a 6-well tissue culture dish with confluent MDCK cells.
2. Aspirate medium and wash cells once with PBS.
3. Remove PBS and infect cells in duplicate wells with 0.5 ml of the 10^{-5}, 10^{-6}, and 10^{-7} virus dilutions in FCS-free MEM medium.
4. Place in a CO_2 incubator at 37°C for 1 hr, rocking the dish at 15-30 min intervals to keep cells moist.
5. Before the 1 hr infection is finished, melt 2% low-melt agarose and place in a 45°C water bath to cool. Be sure it cools to 45°C before using it to overlay cells. Prepare and warm to 45°C MEM medium containing 10%BSA.
6. Mix equal volumes of 2% agarose and the MEM medium from step 5.
7. Aspirate the viral inoculum from the cells (from step 4). Overlay each well with 2 ml agarose and allow to solidify at room temperature (RT) or 4°C. Place in a CO_2 incubator at 37°C for 3 days.
8. Remove the agarose and add 0.5 ml of 0.1% crystal violet to each well. Incubate for 5 min at RT.
9. Aspirate crystal violet and allow wells to dry.
10. Determine the titre by counting plaques within the wells and multiplying by the dilution factor.

PROTOCOL II: PREPARATION OF LIPOSOME

Materials:

- L-α-phosphatidylethanolamines dioleoyl (DOPE, Avanti Polar-Lipids, Inc.)
- 3β[N-(N' N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol, Avanti Polar Lipids, Inc.)

Preparation of 1 ml of Liposome

1. Dissolve DC-chol in chloroform at a concentration of 60.1 mg/10ml and store at –20°C.
2. Dissolve DOPE in chloroform at a concentration of 59.9 mg/10 ml and store at –20°C.
3. Mix 100 µl each of DC-Chol and the DOPE stock solutions.
4. Evaporate it with evaporator with gentle mixing at 40°C.
5. Add 1 ml of HEPES buffer (sterile, pH 7.8) and dissolve the pellet completely with vortex.
6. Hydrate at 4°C for 12 to 72 hrs.
7. Sonicate it at output 2-3, duty cycle 30 (twice for 5 min).
8. Store at 4°C until use.

Synthesis of liposome-DNA mixture

1. Dissolve 5-50 µg of plasmid DNA in 50 µl of PBS.
2. Mix 50 µl of the DNA solution with 50 µl of liposome and pipet several times.
3. Set the liposome-DNA mixture at RT for 15 to 30 min before administration (Do not incubate the DNA with the liposome for more than 1 hr before administration).
4. Administer 100 µl of liposome-DNA mixture per mouse.

PROTOCOL III: FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Probe Preparation

Materials:

- Digoxigenin-11-dUTP (1 nmol/µl, Roche Diagnostics Inc., Mannheim, Germany)
- DNA polymerase I (10 U/µl, GibcoBRL)
- 10 x A4 dNTP mix (Roche Diagnostics Inc., Mannheim, Germany)
- DNA polymerase I (0.5 U/µl)/DNase I (0.4 mU/µl) mix (GibcoBRL)

Probe Labeling

1. Purify PCR product from agarose gel using QIAquick gel extraction kit (Qiagen).
2. Mix following reagents at RT:
   a) 10 x A4 dNTP mix, 5 µl
   b) PCR DNA, 1 µg
   c) Dig-11-dUTP, 1 µl
   d) DNA polymerase I, 1 µl
   e) DNA polymerase I/DNase I mix, 4.5 µl
   f) Make up to 50 µl with H_2O
3. Incubate the mixture at 15^oC for 50 min.
4. Stop the reaction at 80^oC for 10 min.
5. Check the probe on 2% agarose gel (300-560 bp DNA was visible).

Preparation of Specimen Sections

1. Remove the tissue from the animal.
2. Put the tissue in a disposable vinyl specimen mold.
3. Immediately soak specimen mold into liquid nitrogen.
4. Cut block into 10 µm thin sections.
5. Dry slides at RT for 30 min.
6. Wash slides with PBS at RT for 5 min.
7. Incubate slides in 75 mM KCl at RT for 10 min.
8. Soak slides in methanol/acetate acid mix (3 vol:1 vol) at RT for 10 min (to fix tissue).
9. Dry slides at RT for 5 min.
10. Wash slides with PBS at RT for 5 min.
11. Incubate slides with 0.005% trypsin in PBS at 37^oC for 15 min (remove protein).
12. Wash slides with PBS twice at 4^oC for 5 min.
13. Incubate slides with 10 µg/ml of RNase in SSC (degrades endogenous RNA).
14. Wash slides with PBS twice at RT for 5 min.
15. Incubate slides in 4% PFA at RT for 5 min (fix tissue).
16. Wash slides with PBS twice at RT for 5 min.
17. Incubate slides in 0.1% NP-40/2 x SSC at 37^oC for 30 min.
18. Soak slides in 70%, 85% and 100% ethanol at RT for 2 min each (to fix tissue).

Detection with FISH method

Materials:

- RNase: Make 1 mg/ml stock in 2 x SSC. Inactivate DNase by placing in boiling water for 10 min. Freeze in aliquots suitable for 1:10 dilution.
- 20 x SSC (pH 7.00): 3 M NaCl + 0.3 M Na citrate, pH 7; filter before use.
- Master mix (50% formamide, 2 x SSC):
  a) Formamide, 5 ml
  b) 20 x SSC, 1 ml

c) Dextran sulfate, 1 g
d) Must heat for 1-2 hrs to dissolve. Bring to pH 7.0 with HCl. Bring volume to 7.0 ml with ddH_2O.

- Denaturing solution: 70% formamide in 2 x SSC (0.7 volume formamide + 0.1 volume 20 x SSC) pH 7.0 with HCl. Bring to volume with ddH_2O.
- Hybridization wash buffer: Prepare 50% formamide in 2 x SSC (0.5 volume formamide + 0.1 volume 20 x SSC) and adjust pH to 7.0 with HCl. Bring to volume with ddH_2O.
- 4 x SSC/0.1% Triton X-100: Dilute 20 x SSC 1:5, adjust pH to 7.0 and then add Triton X-100 to a final concentration of 0.1%. Autoclave.
- Anti-Digoxigenin-Rhodamine polyclonal antibody (Roche Diagnositic Inc., Mannheim, Germany): Dilute to the final concentration with 5% skim milk in PBS.

Day 1

1. Mix:
   a) Nick product, 40 µl
   b) 3 M sodium acetate, 4 µl
   c) Salmon sperm DNA (10 mg/ml), 4 µl
   d) 100% ethanol, 100 µl
2. Keep at –80°C for 15 min.
3. Centrifuge at 1500 rpm for 30 min in a microcentrifuge.
4. Dry up.
5. Dissolve DNA in 20 µl of a solution containing 70% master mix and 30% ddH_2O.
6. Denature probe mixture at 73°C for 5 min.
7. Place on ice.
8. Soak treated tissue slides at 73°C denaturation solution for 5 min.
9. Incubate tissue slides with 70% ethanol for 2 min on ice.
10. Incubate tissue slides with 85% ethanol for 2 min on ice.
11. Incubate tissue slides with 100% ethanol for 2 min at RT.
12. Dry tissue slides on 37°C hot plate.
13. Incubate with denatured probe mixture.
14. Cover the tissue slides with cover glass and seal with paper cement.
15. Incubate tissue slides at 37°C in a moist incubator for 1-3 days.

Day 2

1. Remove paper cement and cover glass.
2. Wash slides three times at 45°C with 50% formamide/2 x SSC for 10 min.
3. Wash slides once at 45°C with 2 x SSC for 10 min.
4. Wash slides once at RT with 2 x SSC for 10 min.
5. Block with 5% skim milk at RT.
6. Incubate slides with anti-Digoxigenin-Rhodamine Ab (1:200 dilution, protected from light in all of the following steps).
7. Wash slides with 4 x SSC at RT for 10 min.
8. Wash slides with 4 x SSC/0.1% Triton X-100 at RT for 10 min.
9. Wash slides with 4 x SSC at RT for 10 min.
10. Wash slides with H_2O at RT for 5 min.
11. Dry slides.
12. Counter stain slides with 0.05 µg/ml of 4,6-diamidino-2-phenylindole in PBS.
13. Store slides at –4°C until counting.

PROTOCOL IV: ELISPOT ASSAY

Materials:

- Blocking solution: RPMI 1640 containing 10% FCS
- Wash buffer: 1 x PBS containing 0.05% Tween-20
- Plate: MultiScreen-IP sterile Plate, Millipore (Cat. No. MAIPS4510)
- Anti-mouse IFN-γ mAb 1 mg/ml (x 100 in PBS) (PharMingen, Cat. No. 554431)
- Biotinylated anti-mouse IFN-g mAb (x 1000 in PBS) (PharMingen)
- Alkaline phosphatase streptavidin (Vector Laboratories, Inc. CA; Cat. No. SA-5100; working solution: dilute 1 µl of alkaline phosphatase streptavidin in 5 ml of PBS containing 0.05% Tween-20 and 5% BSA just before use)
- BCIP/NBT phosphatase substrate (Kirkegaard & Perry Laboratories, Inc., MD; Cat. No. 50-81-18)

1. Incubate 50 µl/well of 100-fold diluted anti-mouse IFN-γ mAb in PBS to ELIspot IP plate at 4^{o}C overnight.
2. Discard the mAb and wash wells once with 200 µl/well blocking solution.
3. Incubate 200 µl/well blocking solution at RT for 2 hrs.
4. Discard the blocking solution.
5. Add 200 µl/well of 10^4, 10^5 or 10^6 splenocytes with or without antigen 10 µg/ml in RPMI1640 with 10%FCS (each sample was stimulated in triplicate with or without antigen at each dose).
6. Place the plate in a 37^{o}C-incubator at 5% CO_2 for 24 hrs.
7. Discard the cell suspension.
8. Wash wells twice with 200 µl of ddH_2O and allow wells to soak for 3-5 min at each wash step.
9. Wash wells three times with 200 µl/well wash buffer.
10. Discard the wash buffer and incubate 100 µl/well of 1,000-fold diluted Biotinylated anti-mouse IFN-γ mAb at RT for 2 hrs.
11. Wash wells three times with 200 µl/well wash buffer.
12. Discard wash buffer and incubate with 50 µl/well of diluted alkaline phosphatase streptavidin at RT for 1-2 hrs.
13. Wash the plate six times with 200 µl of ddH_2O containing 0.05% Tween-20.
14. Incubate the plate with 50 µl/well of BCIP/NBT phosphatase substrate at RT for 20 min-1 hr.
15. Wash the plate three times with 200 µl of ddH_2O.
16. Dry the plate and count spots with a computer-assisted video image analysis (CVIA) system.