Transplacental Genetic Immunization After Intravenous Delivery of Plasmid DNA to Pregnant Mice[1]

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A number of factors influence the development of tolerance, including the nature, concentration, and mode of Ag 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 Ags from HIV-1 and influenza virus) were administered i.v. to pregnant mice. We examined the uptake of plasmid DNA by the fetuses until the 21st postcoital day, but little such transfer occurred in early pregnancy. At 9.5 days postconception with cationic liposomes, injected plasmid was present in the tissues of the fetus, consistent with transplacental transfer. When the offspring of vaccinated dams were immunized with DNA vaccine, they mounted stronger Ag-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 Ag-specific immunity to their progeny. *The Journal of Immunology,* **2001, 167: 5478–5484.**

Most vaccines intended for human use are administered to infants and children. Due to the immaturity of their immune system, newborns exposed to foreign Ags are at risk of developing tolerance rather than immunity (1–6). For example, if Ag is administered shortly after birth, forbidden clones can emerge and induce such tolerance (1–6). A number of factors influence the development of tolerance, including the nature, concentration, and mode of Ag presentation to the immune system, as well as the age of the host (7, 8). 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. These safety studies enrolled normal adults, but if successful, could broaden to include women of child-bearing age and newborns. Yet there are reports of neonatal tolerance developing when a DNA vaccine encoding the circumsporozoite Ag of malaria was injected into newborn mice (7). There is also a report showing that plasmid DNA can be transmitted to fetuses through the placenta, and that the protein encoded by such a plasmid is synthesized by fetal cells (9).

In the present study, we confirmed that plasmid DNA administered to pregnant mice can reach the fetus through the placenta. This was true both of DNA vaccines encoding the *env* and *gag* genes of HIV-1 as well as those encoding the influenza virus hemagglutinin (HA),[3] matrix (M), and nucleoprotein (NP) genes. Analysis of the immune response of offspring whose mothers were immunized with the influenza DNA vaccine indicates that these progenies had enhanced level of protection against the same virus infection.

Materials and Methods

Animals

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

Viral protein expression plasmids and Abs

pCMV160IIIB encoding the *env* gene of HIV-1 strain IIIB has been described in detail in our previous report (10). pCMVgag was constructed from the *gag* gene of HIV-1 strain IIIB using the same plasmid vector of pCMV160IIIB plasmid. In addition, a plasmid DNA preparation used in the present study contained liposomes (11).

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 (12). 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 (9). DNA vaccines of NP (A/pCMV-V1NP) and HA (V1J-HA(PR8)) (13, 14) genes of the A/PR/8/34 strain (15–17) were kind gifts from J. J. Donelly and D. Montgomery, Merck Research Laboratory (West Point, PA). Anti-HA (C 179) and anti-M1 mAbs (mAb, C111) were purchased from Takara Biomedicals (Kyoto, Japan). To confirm that plasmid DNA was transferred through the placenta, a *lac*Z expression plasmid containing a chicken β-actin promoter was also used.

Virus

Mouse-adapted influenza A/PR/8/34 (H1N1) viruses were used in this study. Viruses were harvested from infected Maudin-Darby canine kidney cells and titrated according to the plaque formation method.

DNA immunization

Mothers were injected i.v. with DNA vaccine before or after coitus. Preparations containing various doses of the DNA vaccine were encapsulated

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[3] Abbreviations used in this paper: HA, hemagglutinin; DTH, delayed-type hypersensitivity; FISH, fluorescence in situ hybridization; M, matrix; NP, nucleoprotein; p.c., postconception.

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into liposomes (11). Briefly, a mixture of 3β-[*N*′-(*N*′-dimethylaminoethane) carbamoyl] cholesterol and dioleoylphosphatidylethanolamine in chloroform was dried, vacuum desicated, resuspended in sterile HEPES buffer (pH 7.4), and used for the cationic liposomes. Before administration, an appropriate amount of DNA in 0.15 mol PBS (pH 7.2) was mixed with the liposome solution at a volume ratio of 17:3. The pregnant mice were i.v. administered the DNA vaccine or empty vector on various days after postconception (p.c.) to assess immunogenicity. Six weeks after birth, their offspring were injected 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 HA and NP protein was administered into pregnant mice by i.v. route. The same amount of DNA plasmids were administered to offspring by intranasal route.

Slot blot analysis

The conventional method for detecting DNA was performed using slot-blot analysis described elsewhere (9).

Fluorescence in situ hybridization (FISH) analysis

FISH analysis was performed essentially according to the method of El-Naggar et al. (18). A 564-bp HIV *env* region fragment (nt 1569–2133) amplified from pCMV160IIIB plasmid (10) was labeled with digoxigenin-11-dUTP by nick translation and used for a hybridization probe. The tissue samples were taken from mice to which 50 μg HIV plasmid pCMV160IIIB (10) or influenza plasmid V1J-HA(PR8) (14, 15) with liposomes was administered and sliced to prepare histological examination. The samples, which were deparaffinized in xylene and ethanol, were denatured and hybridized with digoxigenin-labeled probes using previously described method (18). After hybridization, the slides were washed and stained with an antidigoxigenin rhodamine (a red fluorochrome; Boehringer Mannheim, Mannheim, Germany). The slides were counterstained with 4,6-diamino-2-phenylindole dihydrochloride. Images were made with a Nikon SA fluorescence microscope (Nikon, Tokyo, Japan) and a charge-coupled device camera interfaced with a Cyto Vision (Applied Imaging, Sunderland, U.K.).

RT-PCR

Total RNA was isolated using TRIzol (Life Technologies, Grand Island, NY) by means of previously described methods (19, 20). Briefly, quick frozen samples were thawed and extensively treated with DNase. Then RNA was converted to cDNA using a GeneAmp RNA PCR kit (PerkinElmer, Norwalk, CT). The total RNA was reverse transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase. As a control experiment, we tested RNase treatment before reverse transcription. For detecting the HIV-1 *env* gene, cDNA was amplified in a 30-cycle PCR using primers GGAGCAGCAGGAAGCACTAT and TCCCT GCCTAACTCTA. The primers for nested PCR were TGCTGAGGGCTA TGA and GCCTCCTACTATCATTATG. For detecting β-actin gene, the primers TGGAATCCTGTGGCATCCATGAAAC and TAAAACGCAGCT CAGTAACAGTCCG were used.

Recombinant proteins and peptides

The DNA fragments encoding almost the full length of the influenza M and NP genes were amplified from pME18S-M and A/pCMV-V1NP by PCR. The PCR products were inserted into pGEX-3X (Pharmacia Biotech, Uppsala, Sweden) by directional cloning. pGEX-M and pGEX-NP were transformed into *Escherichia coli* JM 101. Purified influenza M gene product and NP protein were obtained using a bacterial expression system. Recombinant gp160 from HIV-1IIIB was obtained from MicroGene System (Meriden, CT). HGP30 peptide (YCVHQRIEIKDTKEALDKIEEEQNK SKKKA) was used for detection of the HIV-1 *gag* immune response (21, 22); and influenza M1 peptide (KAVKLYRKLKRE) was used as an Ag for ELISA. We also included mice that were i.m. injected inactivated influenza A/PR/8/34 as a control Ag. For the CTL assay, we used HIV-1 V3 peptide (RGPGRAFVTI) (23) to synthesize the Ag as well as the peptide ALVEADVA of sperm whale myoglobin (24) as a control peptide.

Delayed-type hypersensitivity (DTH) response

Seven days after i.m. immunization with 20 μg of the same expression plasmids as received by their mothers, a total of 25 μl of PBS containing 5 μg of gp160, HGP-30 peptide, influenza M gene product, or heat-killed A/PR/8/34 virus was injected into the right footpad of immunized offspring. After 24 h, the extent of footpad swelling was measured by previously described method (25).

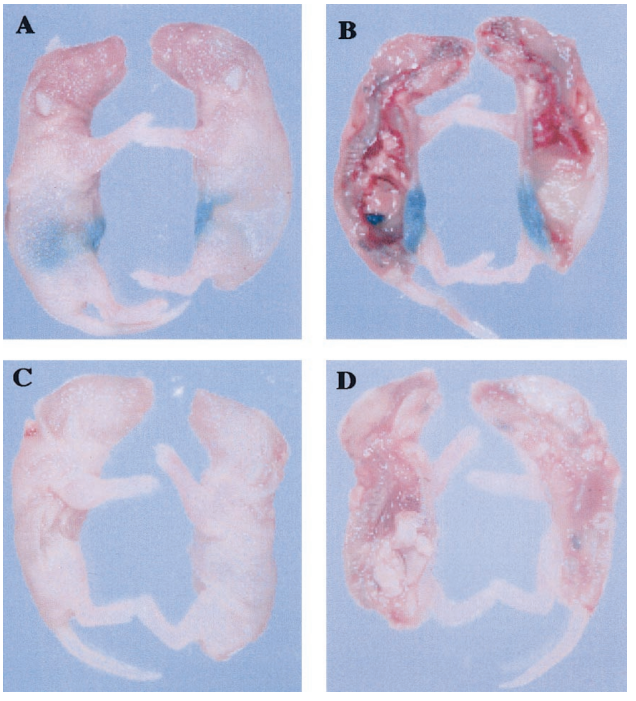

FIGURE 1. Gene expression of *lacZ* in mouse fetus. *A* and *B*, Stained tissue of a fetus cut in two, whose mother received 30 μg of *lacZ* plasmid with liposomes at day 9.5 postcoitus (p.c.); *C* and *D*, stained tissue of a fetus whose mother received 30 μg of empty plasmid with liposomes at day 9.5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

ELISA

ELISA was used for titration of serum Ag-specific IgG, IgG1, IgG2a, and IgM responses. Offspring of both immunized and nonimmunized mothers were bled at 7 days after i.m. immunization with various DNA vaccines, as described elsewhere (26). A total of 10 μg/ml gp160 protein, HGP-30 peptide, rNP protein, M1 synthetic peptides, or heat-killed A/PR/8/34 virus was coated on 96-well microplates (Nunc, Roskilde, Denmark); after blocking with 3% BSA in PBS, serially diluted antisera were added and incubated at 37°C for 2 h. Peroxidase-conjugated goat anti-mouse IgG (Organon Teknika, West Chester, PA) was used as the secondary Ab; then

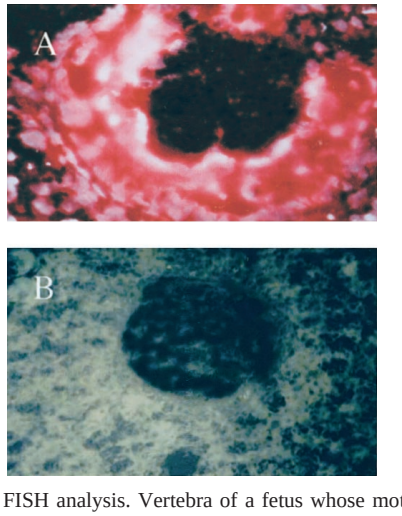

FIGURE 2. FISH analysis. Vertebra of a fetus whose mother received HIV plasmid pCMV160IIIB (*A*) or influenza plasmid V1J-HA(PR8) (*B*) with liposomes at day 9.5 p.c. These samples were reacted with HIV *env* region fragment labeled with digoxigenen-11-dVTP, followed by staining with an antidigoxigenin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

Table I. *Ab response of the progeny after i.v. injection of pregnant mice with expression plasmids*[a]

Immunogens	Reciprocal Log_2 Titer				
	gp160	HGP-30 peptide	A/PR/8/34	NP protein	M1 peptide
pCMV160IIIB (50 μg)	9.6 ± 1.0[b]	<2	<2	NT	NT
pCMV160IIIB (10 μg)	6.5 ± 1.1[b]	<2	<2	NT	NT
pCMV160IIIB (1 μg)	5.7 ± 0.4	<2	NT	NT	NT
Empty vector (50 μg)	4.3 ± 0.7	NT	NT	NT	<2
pCMV160IIIB (50 μg)[c] (without liposome)	7.2 ± 1.3[b]	<2	<2	NT	<2
pCMV gag (50 μg)	<2	10.8 ± 2.0[b]	NT	NT	NT
Empty vector (50 μg)	<2	4.0 ± 0.4	NT	NT	NT
pCMV gag (50 μg)[c] (without liposome)	<2	7.4 ± 1.8[b]	NT	NT	NT
pCMV gag pol (50 μg)	<2	9.0 ± 1.4[b]	NT	<2	NT
Empty vector (50 μg)	<2	4.8 ± 0.3	NT	<2	NT
V1J-HA(PR8) (50 μg)	NT	<2	10.7 ± 1.7[b]	NT	2.2 ± 0.3
Empty vector (50 μg)	NT	<2	5.3 ± 1.0	NT	1.9 ± 0.4
A/pCMV-V1NP (50 μg)	NT	<2	NT	6.7 ± 0.9[b]	<2
Empty vector (50 μg)	NT	<2	NT	4.2 ± 0.6	NT
pME18S-M (50 μg)	NT	NT	NT	NT	8.7 ± 1.4[b]
Empty vector (50 μg)	NT	NT	NT	NT	4.2 ± 0.3

[a] At day 9.5 p.c., pregnant BALB/c mice were i.v. injected with each expression plasmid, or empty vector, and liposomes.
[b] Significant difference ($p < 0.05$) from the empty vector control group. NT, not tested. Two other separate experiments showed similar results.
[c] Fifty milligrams of pCMV160IIIB was i.v. injected without liposomes. Six weeks after birth, their progeny were immunized i.m. with 50 μg of the same expression plasmids without liposomes as received by mothers. The offspring whose mothers received empty vector were also immunized with the expression plasmids of the same group. After 7 days, blood was collected and the Ab titers were detected by ELISA using each gp160, HGP-30 peptide, heat-killed A/PR/8/34 virus, NP, or M1 peptide. Data represent means ± SE of five to eight mice.

the plates were stained with 3,3′,5,5′-tetramethylbenzine (DAKO, Carpenteria, CA). For Ag-specific IgG1, IgG2a, and IgM responses, HRP-coupled anti-mouse IgG1, IgG2a, or IgM (Organon Teknika) was used as the secondary Ab. Specific Ab titers were expressed as the reciprocal value of the final dilution, which gave an OD (A_{415}) of ≧ 0.2 OD units compared with each preimmunized sample.

Cytokine ELISPOT assay

The cytokine ELISPOT assay was performed with minor modifications, as previously described (26, 27). Briefly, 96-well microplates (MAIPS4510; Millipore, Bedford, MA) were coated with anti-mouse IFN-γ rat mAb (BD PharMingen, San Diego, CA), 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 V3 peptide. After a 48-h culture, plates were washed and incubated again for 1 h with biotinylated anti-mouse IFN-γ mAb (BD PharMingen). Then, after peroxidase staining, the spots in each well were counted, using the dilution to calculate the total number of cytokine-secreting cells utilizing a computer-assisted video image system (Zeiss, Oberkochen, Germany).

HIV-1-specific CTL response

Mother mice that received pCMV160IIIB were immunized with 50 μg of the same plasmid with liposomes, and spleen cells were collected. The details of the CTL assay have been described previously (28). We also obtained spleen cells from offspring whose mothers were injected with the empty vector. Then 1×10^6 lymphoid cells were restimulated in vitro in the presence of the same amount of irradiated (30 Gy) syngenic spleen cells with 5 μg/ml HIV-1 V3 peptide RGPGRAFVTI (29, 30), a known CTL epitope of HIV-1IIIB. After a 5-day culture, the cytotoxic activity of these spleen cells was measured by a 6-h ^{51}Cr release assay using V3 or myoglobin peptide-pulsed target P815 (H-2^d) cells.

Virus challenge

Under light diethylether 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 ANOVA for distribution parameters. Significance was defined as $p < 0.05$ in both analyses.

Table II. *Effect of timing of DNA vaccination of mothers on Ab responses of their progeny*[a]

Group	Timing of DNA Vaccination into		Reciprocal Log_2 Titer
	Mothers	Progeny (6 wk)	gp160 protein
1	2 wk before coitus (pCMV160IIIB)	pCMV160IIIB	4.1 ± 1.2
2	0–3 wk p.c. (pCMV160IIIB)	pCMV160IIIB	6.0 ± 0.7
3	5–8 wk p.c. (pCMV160IIIB)	pCMV160IIIB	8.2 ± 0.8*
4	9–11 wk p.c. (pCMV160IIIB)	pCMV160IIIB	10.9 ± 1.6*
5	12–18 wk p.c. (pCMV160IIIB)	pCMV160IIIB	10.5 ± 1.2*
6	10 wk p.c. (empty vector)	pCMV160IIIB	4.2 ± 1.5
7	10 wk p.c. (empty vector)	Empty vector	2.4 ± 0.3

[a] Mice at various stages of pregnancy were i.v. injected with 50 μg of pCMV160IIIB, or empty vector, with liposomes. In one group, the mothers were i.v. administered pCMV160IIIB at 2 wk before coitus. Six weeks after birth, all offspring except those of group 7 were immunized i.m. with 50 μg of the same expression plasmids as received by their mothers. In a control group, mothers and offspring were injected i.m. with empty vector only. After 7 days, blood was collected and the Ab titers were detected by ELISA using gp160.
[b] Significant difference compared with group 6 ($p < 0.05$). Data represent means ± SE of four to eight mice.

Table III. *Types of Ab response of offspring after i.v. injection of pregnant mothers with pCMV160IIIB or pCMV-V1NP*[a]

	Immunization of		Reciprocal Log_2 Titer					
			IgG1		IgG2a		IgM	
Group	Pregnant mothers	Progeny	Before immunization	After immunization	Before immunization	After immunization	Before immunization	After immunization
1	pCMV160IIIB	pCMV160IIIB	4.1 ± 0.6	10.3 ± 1.3[b]	3.0	6.0 ± 0.9	4.3 ± 1.1	6.7 ± 1.8
2	Empty vector	pCMV160IIIB	<2	5.4 ± 0.7	<2	5.3 ± 1.2	<2	5.2 ± 0.4
3	pCMV-V1NP	pCMV-V1NP	4.5 ± 0.8	8.9 ± 2.4[b]	2.7	5.5 ± 0.7	4.6 ± 0.4	5.0 ± 0.4
4	Empty vector	pCMV-V1NP	<2	5.9 ± 0.9	<2	5.0 ± 0.7	<2	5.3 ± 0.2
5	Empty vector	Empty vector	<2	<2	<2	<2	<2	<2

[a] At day 9.5 p.c., pregnant BALB/c mice were i.v. injected with 50 μg of pCMV160IIIB, pCMV-V1NP, or empty vector, with liposomes. Six weeks after birth, progeny of groups 1 and 2 were immunized i.m. with 50 μg of pCMV160IIIB. In groups 3 and 4, offspring were injected i.m. with pCMV-V1NP with liposomes. Two days before immunization, the blood samples were collected (before immunization). After 7 days, blood samples were collected (after immunization) and Ab titers were detected by ELISA using gp160 or NP, respectively. Data represent means ± SE of six to eight mice.

[b] Significant difference ($p < 0.05$) from the empty vector control.

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 used. To increase the uptake of this plasmid, it was liposome encapsulated before i.v. delivery (7). 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.

Slot-blot analysis of offspring whose mothers had received liposome-encapsulated plasmid DNA-encoding genes from HIV was performed as previously described (9). There was extensive evidence of plasmid DNA reaching these fetuses of mothers injected at 9.5 and 14.5 days of gestation (data not shown). In a separate experiment using densitometric analysis, when mice immunized with pCMV160IIIB and liposomes 9.5 days p.c. were compared with those immunized with pCMV160IIIB only, it was found that the uptake in those treated with liposomes was 3.2 times that in those that were not so treated. 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). Further evidence of extensive plasmid transfer was provided by RT-PCR analysis of tissues from newborn mice. mRNA of the HIV-1 *env* gene could be detected in mice whose mothers had received the DNA vaccine from 5.5 to 17.5 days p.c. (data not shown).

Immune responses of mice born from plasmid DNA-vaccinated dams

The offspring of mice vaccinated while pregnant with one of several different DNA plasmids were immunized at 6 wk of age with the same plasmid. Ab titers among offspring of vaccinated mothers were significantly higher than those of mothers injected with vector alone (Table I). Improved Ab responses were seen for a variety of epitopes encoded by HIV-1 and influenza genes. The Ab titers of mice at 6 wk of age whose mothers received plasmid DNA were below 2 when we tested ELISA using each corresponding Ag (data not shown). The timing of maternal DNA vaccination on the immune response of offspring was then examined. Mothers were immunized either 2 wk before being mated, or at various periods p.c. At 6 wk postbirth, the offspring were vaccinated and their Ab response was tested after 7 days. Vaccination before mating had no

Table IV. *Ag-specific DTH responses of progeny after i.v. injection of pregnant mice with various expression plasmids*[a]

	Footpad Swelling Response ($\times 10^{-3}$ cm)							
	gp160		HGP-30		A/PR/8/34		M1 peptide	
Immunogens	Before immunization	After immunization	Before immunization	After immunization	Before immunization	After immunization	Before immunization	After immunization
pCMV160IIIB[b] (50 μg)	3.2 ± 1.0	7.6 ± 1.1	NT	NT	NT	2.0 ± 0.2	NT	NT
pCMV160IIIB (50 μg)	4.7 ± 1.2	15.3 ± 3.1[c]	NT	NT	NT	1.6 ± 0.7	2.6 ± 0.6	1.8 ± 0.4
pCMV160IIIB (10 μg)	4.6 ± 0.2	9.8 ± 0.4[c]	NT	NT	NT	2.2 ± 0.4	NT	NT
pCMV160IIIB (1 μg)	4.9 ± 0.3	10.7 ± 0.6[c]	NT	NT	NT	1.4 ± 0.3	NT	NT
Empty vector (50 μg)	2.5 ± 0.4	10.4 ± 1.9	NT	NT	NT	1.7 ± 0.5	NT	NT
pCMV gag (50 μg)	NT	2.4 ± 0.3	3.5 ± 0.5	14.6 ± 3.2[c]	NT	2.3 ± 0.6	NT	NT
Empty vector (50 μg)	NT	NT	2.0 ± 0.4	9.2 ± 1.3	NT	5.2 ± 0.6	NT	NT
V1J-HA(PR8) (50 μg)	NT	2.2 ± 0.2	NT	2.5 ± 0.6	4.1 ± 0.6	14.2 ± 1.7[c]	NT	1.7 ± 0.5
Empty vector (50 μg)	NT	NT	NT	1.8 ± 0.3	2.3 ± 0.5	6.5 ± 1.0	NT	1.3 ± 0.2
pME18S-M (50 μg)	NT	NT	NT	NT	NT	1.9 ± 0.3	4.2 ± 0.8	14.8 ± 2.7[c]
Empty vector (50 μg)	NT	0.9 ± 0.1	NT	2.2 ± 0.6	NT	2.1 ± 0.3	1.4 ± 0.4	6.0 ± 0.4

[a] At day 9.5 p.c., pregnant BALB/c mice were i.v. injected with each expression plasmid, or empty vector, and liposomes. Six weeks after birth, their progeny were immunized i.m. with 20 μg of the same expression plasmids with liposomes as their mothers received (after immunization).

[b] About 2 wk before coitus, the mothers were administered 50 μg of pCMV160IIIB and liposomes, and their offspring were immunized i.m. with 20 μg of pCMV160IIIB. After 7 days, these mice were challenged with 5 μg of gp160, HGP-30 peptide, heat-killed A/PR/8/34 virus, and M1 peptide, respectively. Data represent means ± SE of five to nine mice.

[c] Significant difference ($p < 0.05$) from the empty vector control group. Two other separate experiments showed similar results.

Table V. *ELISPOT analysis of IFN-γ-producing spleen cells from DNA-vaccinated mice whose mothers had been injected with the same vaccine during pregnancy*[a]

Immunogen Administered to		IFN-γ-Producing Cells (spot/10^6 spleen cells)
Pregnant mother	Progenies	
Group 1: pCMV160IIIB	pCMV160IIIB	42.6 ± 9.6[b,c]
Group 2: pCMV160IIIB	Nonimmune	20.6 ± 6.7[c]
Group 3: Empty vector	pCMV160IIIB	28.2 ± 3.5*
Group 4: Empty vector	Empty vector	16.7 ± 6.3
Group 5: Nonimmune	Nonimmune	13.9 ± 5.9

[a] 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 2 days. Data represent means ± SE of six to eight mice.

[b] Significant difference ($p < 0.05$) from the empty vector control.

[c] Significant difference between these two. The data of two other experiments showed similar results.

effect on the immune response of the litter (Table II). By comparison, significantly Ag-specific responses were observed in the offspring of mice vaccinated from 5 to 18 days p.c. In general, the later vaccination was delayed p.c., the stronger the immune response mounted by the immunized offspring.

The isotype of Ab produced by vaccinated offspring of immunized mothers was examined. There was a significant increase in Ag-specific IgG1, but not IgG2a or IgM titers in these mice (Table III). To provide a measure of Ag-specific T cell immunity, offspring of DNA-vaccinated mothers were immunized at 6 wk of age with the same vaccine, and then challenged in the hind footpad 1 wk later with 5 μg vaccine-encoded Ag. Significant enhancement of Ag-specific footpad-swelling response was observed in the offspring of dams vaccinated with 50 μg plasmid DNA (Table IV). Lower doses of maternal vaccination were less effective. Of note, vaccinating either the father or mother before conception did not alter the immune response of the offspring (data not shown). These results suggest that the offspring of vaccinated dams develop improved DTH responses as well as Ab responses by this technique. Confirming the antigenic specificity of this effect, mice challenged with the wrong Ag manifest normal rather than heightened footpad-swelling response (Table IV).

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

The CTL response was then investigated and found to be enhanced in offspring whose mothers had received DNA vaccine. In mice of mothers treated with DNA vaccine, DNA plasmids were transmitted into the fetuses through the placenta, resulting in the induction of a stronger CTL response (Fig. 3).

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 wk of age. Seven days later, they were challenged with influenza virus A/PR/8/34. Whereas few normal mice survived challenge, >70% of the offspring of vaccinated mothers survived (Fig. 4).

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 mother vaccinated 20 days before mating) survived challenge, >50% of the offspring of immunized mothers survived (Table VI).

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 postvaccination.

Discussion

Three independent techniques were used to establish that plasmid DNA administered to pregnant mice could reach the fetus. First, administration of a β-galactosidase-encoding plasmid allowed for the direct identification of protein expression in neonates. Although protein levels were low (they were undetectable by Western blot analysis), clear detection of multiple organs and intense staining of the placenta were consistent with transplacental migration of the plasmid. Second, RT-PCR allowed identification of mRNA encoded by the DNA vaccine in fetal tissues, especially those from mothers injected after day 9.5 p.c. DNA vaccination in the precoital period or before day 3 of pregnancy did not result in detectable fetal transmission of the plasmid, presumably due to

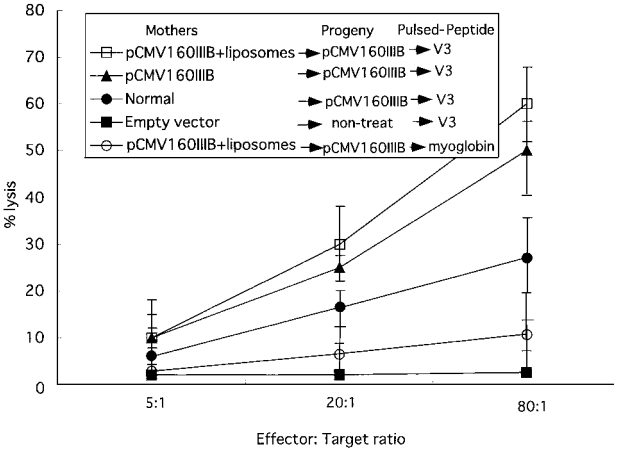

FIGURE 3. CTL response of immune BALB/c mice, whose mothers had been administered with the same DNA vaccine. Day 9.5 p.c. pregnant BALB/c mice were i.v. injected with 50 μg of pCMV160IIIB with (□) or without (▲) liposomes. Six weeks after birth, some offspring were immunized i.m. with 50 μg of pCMV160IIIB. Progeny of mothers that received only empty vector were immunized i.m. with pCMV160IIIB (●). Normal nonimmune mice were used as a control group (■). After 7 days, spleen cells were collected and cocultured for another 5 days with V3 peptide-pulsed P815 cells. As a control, P815 cells pulsed with the myoglobin peptide ALVEADVA were also used (○). Data represent means ± SE of four to six mice. Spleen cells from mice immunized twice with 20 μg of myoglobin with CFA lysed 42.3% of the myoglobin-pulsed target cells at an E:T ratio of 80:1.

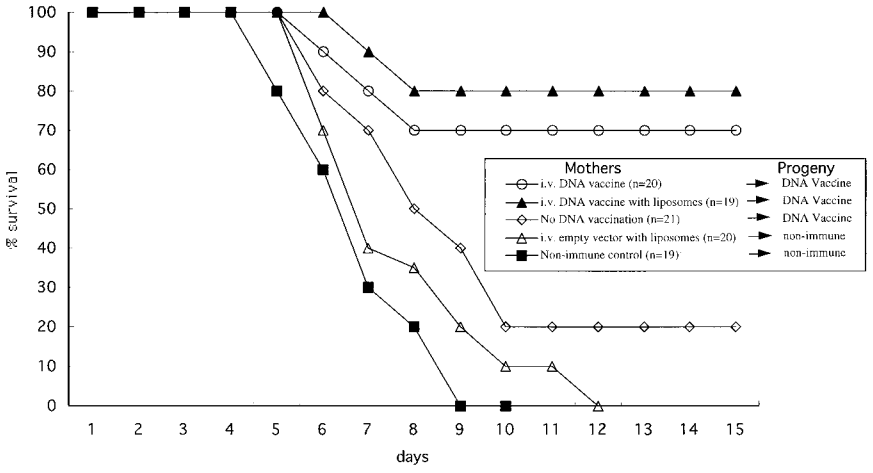

FIGURE 4. Protection of offspring against a lethal A/PR/8/34 influenza virus challenge. Day 9.5 p.c. pregnant BALB/c mice were i.v. injected with 25 μg each of pME18S-M and pCMV-V1NP with (▲) or without (○) liposomes. Six weeks after birth, their offspring were immunized i.m. with a total of 50 μg of 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 (△). Nonimmunized normal mice were used as the other control (■). After 7 days, all mice were challenged with 5 LD_{50} A/PR/8/34 virus. The percentage of survival of these mice was studied for another 15 days. *n*, Number of mice.

immaturity of the placenta at this early stage (Table II). Third, the FISH method was used to directly detect plasmid DNA in fetal tissue (Fig. 2). Our results confirm and extend the previous finding (9) that a β-galactosidase plasmid can be transmitted through the placenta to the fetus in mice. Of particular importance, we established that such transplacental transfer influences the recipients' subsequent capacity to mount an immune response against the plasmid-encoded Ag. This was manifested both by improved humoral and cellular immunity (Tables I-V) and higher levels of pathogen-specific protection (Fig. 4 and Table VI).

These studies were also performed to test the hypothesis that the administration of a DNA vaccine during pregnancy may induce Ag-specific tolerance in the offspring, as suggested by the clonal selection theory of Burnet (2, 3, 31). 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 (Tables I-IV). Using this technique, we did not observe Ag-specific immune tolerance in progeny, as reported by Mor et al. (32). This could reflect our use of a different plasmid (Mor detected tolerance following neonatal immunization with a plasmid encoding the circumsporozoite protein of malaria), or the very limited amount of plasmid actually transferred transplacentally. Indeed, Ichino et al. (7) demonstrated that neonatal tolerance was dose dependent, and could be reliably induced only when >10 μg plasmid was injected into newborn mice. This is consistent with other reports showing that low dose Ag can induce immune responsiveness, while high dose immunization can induce tolerance in young recipients (8, 33, 34), which might be supporting Burnet's theory that high levels of neonatal Ag can trigger clonal deletion (3, 7).

When offspring of vaccinated mothers were immunized at 6 wk of age with the same DNA vaccine, they manifested significant anamnestic responses (Table III). Reimmunization was required, however, since transplacental transport of plasmid alone did not trigger strong immune responses in the newborn, nor provide adequate protection from infection (Fig. 4 and Table VI). Yet reexposure of these mice to vaccine at 6 wk of age elicited a strong, protective immune response, characterized by Ag-specific Ab, CTL, and cytokine responses. The administration of DNA vaccine into amniotic fluid induced a high level of protective immunity (35).

We used liposomes throughout our studies, because we could observe high levels of immune responses (Table I and Fig. 4) when we added liposomes as an adjuvant. The same observation was reported previously (9).

Results indicate that the transmission of serum IgG from immunized mothers to offspring seems not to have a major influence on the production of specific Ab at 6 wk of age. A lack of maternal contribution to the DTH response of offspring is particularly evident (Table IV). Thus, while we cannot rule out the possibility that

Table VI. *The importance of timing of DNA immunization of pregnant mothers against A/PR/8/34 virus challenge*[a]

DNA Vaccination of		Survival After Immunization
Pregnant mothers	Progeny	Survivors/total (%)
Group 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)[b]
Day 14.5 p.c.	DNA vaccination	12/19 (63.2)[b]
Day 9.5 p.c.	No vaccination	3/18 (16.7)[b]
Group 2		
Empty vector day 9.5 p.c.	DNA vaccination	2/22 (9.1)
Group 3		
Nonimmune control	Nonimmune	1/19 (5.3)

[a] Pregnant BALB/c mice were i.v. injected with 25 μg each of A/pCMV-V1NP plus 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 5 LD_{50} of A/PR/8/34 and the percent survival was determined after another 20 days. Data represent means $\pm$ SE of 19–22 mice.

[b] Statistically significant difference ($p < 0.05$) among nonimmune control group. The data of another separate experiment showed similar results.

some maternal contribution persisted, only following direct immunization of offspring at 6 wk of age was clear evidence of improved protective immunity observed (Table VI). However, 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, and various other infections occurring in infants as well as animals.

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