Efficient Protection of H5N1 Influenza Virus DNA Vaccine Delivering by Electroporation in Mammalian and Avian System

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Abstract To investigate the protection effect of DNA vaccine in mammalian and avian systems, the DNA vaccine was inoculated in both BALB/c mice and SPF chickens immunized with DNA vaccines encoding hemagglutinin (HA) from A/Goose/GuangDong/1/96 (H5N1) virus. The mice and chickens were immunized twice, 3 weeks apart, by electroporation into muscles or intramuscular injection. Two weeks after the second immunization, the mice and chickens were challenged with a lethal dose of homologous virus. The mice and chickens immunized by electroporation obtained completely protection against the virus, and could effectively inhibited viruses to replicating in mouse lung and chicken cloaca. At the same time, these protections were companied by high levels specific antibody to H5N1 AIV, while the blank plasmid controls experience 100 percent mortality following challenge. Furthermore, in the experiment of mice by eletroporation, stronger obviously CTL activity were observed after challenge. Thus, the cellular immune responses of the mice immunized by electroporation were exhibited. These results strongly demonstrate that HA DNA vaccines provide effective protection against influenza virus infection in mammalian and avian, and suggest that electroporation is one of the efficient gene delivery systems for the transfer of influenza DNA vaccine in both humoral immunity and cellular immunity.

Key words H5N1 AIV, DNA vaccine, immunized by electroporation

Avian influenza virus can cause serious disease in a wide variety of birds and mammals, from symptomless infection to various respiratory disorder, decrease in production, or a rapidly fatal systematic disease. Especially the highly pathogenic avian influenza, which is caused by subtypes H5 and H7, is a catastrophic disease of the poultry industry[1].

Traditionally, it had been thought that H5N1 transmission to human beings would require an intermediate such as the pig, whose respiratory epithelium shares sialic acid isoforms with both birds and human beings[2]. Any doubt that avian influenza A viruses can cross naturally into mammals and cause severe disease was removed by the outbreak in Hong Kong, China in 1997. A highly pathogenic H5N1 virus that circulated in domesticated birds and it infected at least 18 people and caused 6 deaths[3,4]. Furthermore, more than 100 million birds have either died or been culled because of a highly lethal H5N1 avian virus, which has been proved to be responsible for 34 reported human illnesses and 23 deaths, according to World Health Organization (WHO) [5], from mid-December 2003 to April 2004 in Asia. In the same year, H7N7 avian influenza, another virulent subtype, broke out of poultry industry in the Netherlands, and a veterinarian died [6]. These facts demonstrated that highly pathogenic avian influenza A viruses can transmit directly from chicken to human without intermediate mammalian host and cause human infection and death. Influenza is believed the next worldwide pandemic disease[7].

In the past decades, DNA vaccine provides a promising new approach for immunization against influenza viruses. Previous studies have demonstrated that plasmid DNA encoding hemmaglutinin (HA) or nucleoprotein (NP) of influenza virus, which was administrated by a gene gun or intramuscular injection, could elicit specific immune responses and provide protection against influenza in animal models [8~14]. Moreover, a new method was applied to transfer the

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plasmid DNA into muscle by electroporation *in vivo*, which is more efficient compared to simple intramuscular injection[15~19].

To prevent the influenza disease from mammalian host and avian, we inserted HA gene from A/Goose/GuangDong/1/96 (H5N1) virus , which is closely homologous to A/HongKong/ 156/97 (H5N1) with 98% and 98.2% similarity in nucleotide and amino acid sequence, respectively, without a potential glycosylation site at residue 154 of HA [20], into eukaryotic expression vectors pcDNA4/HisMax and pRc/CMV, and inoculated mice and chickens with DNA vaccine of pC4H5 and pCMVH5 by electroporation. We then examined several aspects of immune responses after immunization. Hundred percent of the mice and chickens immunized with pC4H5 or pCMVH5 by electroporation were protected from challenge with homologous virus with high level of antibody and CTL responses. The results clearly demonstrate that the recombined plasmids could protect mice and chickens from virus challenge.

1 Materials and methods

1.1 Animals and virus

BALB/c female mice (4 weeks old) were purchased from Academy of Military Medical Sciences Experimental Animal Centre, and SPF chickens (3 weeks old) were purchased from Experimental Animal Centre (Beijing, China). High pathogenic avian influenza virus A/Goose/ GuangDong/1/96 (H5N1) was used in this study. The BALB/C mice and SPF chickens were always raised in the Gloveboxes and Isolators Class Ⅲ. The highly pathogenic avian influenza were operated under the condition of Biosafety Level 3 (BSL3) laboratory.

1.2 Plasmid DNA

A full-length cDNA copy of HA gene from H5N1 influenza virus A/Goose/GuangDong/1/96 was cloned into the *Eco*R Ⅴ and *Xho* Ⅰ sites of pcDNA4/HisMax, a vector that contains a QBI sp163 transcription enhancer and a cytomegalovirus (CMV) immediate-early promoter, and this construct was designated pC4H5 [21]. The same HA gene was cloned into the pRc/CMV vector under the control of CMV immediate-early promoter as previously described and designated pCMVH5 [21]. The expression of HA-encoded protein was confirmed in HeLa cells, which had bioactivity by hemagglutination assays, as described previously[21]. pcDNA4/HisMax were used as control plasmids for A/Goose/GuangDong/1/96 challenge.

1.3 Immunization by *in vivo* electroporation (E.P.)

In vivo electroporation was carried out according to the method described by Aihara and Miyazaki [15]. BALB/c female mice were immunized by two injections under light anesthesia (pentobarbital sodium, 50 μg/g), 3 weeks apart, of 30 μg /mice into the right quadriceps muscle. A pair of electrode needles with 5 mm apart was inserted into the muscle to cover the DNA injection sites and electric pulses were delivered using an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, CA). Four pulses of 100V each were delivered to each injection site at the rate of one pulse per second, each pulse lasting for 40 ms. Then four pulses of the opposite polarity were applied, as described previously [22].

SPF chickens were immunized using electroporation by two injections, 3 weeks apart, of 30 μg or 50 μg per group of chickens. The other references were the same as immunized to mice.

1.4 Immunization by intramuscular (I.M.) injection

Mice were immunized in each quadriceps muscle with either 30 μg plasmid DNA two times, three weeks apart, under light anesthesia (pentobarbital sodium, 50 μg/g). The concentration of plasmid DNA was 1 g /L.

SPF chickens were immunized with the same method, but without anesthesia.

1.5 Hemagglutinin inhibition (HI) assay

The HI assay of HA activity was performed with 1% chicken red blood cells as previously described [23]. Sera from mice and chickens were tested individually after treatment with receptor-destroying enzyme from cholera vibrio extracts. HI titers were determined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

1.6 Protection assay

Two weeks after the secondary immunization, the mice were challenged intranasally with lethal dose of $10^{5.8}$ ELD_{50} of HPAIV A/Goose/GuangDong/1/96 (H5N1) under light anesthesia (pentobarbital sodium, 50 μg/g). The mice were monitored daily for weight loss, clinical signs, and mortality. Lungs of 3 mice were taken from each group on 5 d, 7 d, 16 d after challenge to detect virus replication.

A couple of weeks after the secondary immunization, the chickens were challenged with lethal dose of $10^{4.2}$ ELD_{50} of HPAIV GD/96(H5N1) by intramuscular injection. After virus challenge, the clinical signs and mortality of chickens were

monitored, and samples in cloaca were taken from five or six from each group on day 5 postinfection for virus replication.

1.7 CTL assay

Splenic CTL activity was determined following a 5-day-stimulation *in vitro* with H5N1-sensitized autologous splenocytes against H5N1-sensitized P815 cells at effect : target ratios of 100:1, 33:1, 10:1, and 3.3:1, according to the method of Kadowaki *et al*[16].

Briefly, spleen cells (5×10^6) were suspended in RPMI medium 1640 containing 10% FCS. The spleen cell suspension was incubated with 10^6 PFU inactivated H5N1 virus for 5 d. At the end of the incubation period, the cells were washed, counted and resuspended at 2×10^7 cells/ml, 6.6×10^6 cells/ml, 2×10^6 cells/ml and 6.6×10^5 cells/ml, respectively, as the effector cell population. P815 cells, as the target cells, were incubated with $10^{7.5}$ EID_{50} of the H5N1 virus at 37℃ for 1 h. The H5N1-infected P815 cells were resuspended at 2×10^5 cells/ml, and 50 μl of cell suspension was added into a 96-well round-bottomed microtiter plate containing triplicate 50 μl samples of the effector cells. The microtiter plate was incubated at 37℃ for 6 h and centrifuged. To detect the cytotoxic T lymphocytes activities, Cytotox96® Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, USA) was used. Specific lysis was calculated from the LDH release using the following formula: % specific lysis = (experimental value−spontaneous effector cells value−spontaneous target cells value)/(target cells maximum release value−target cells spontaneous release value) × 100. These respective references were calculated following the protocol of the manufacturer.

1.8 Statistical analyses

All values were expressed as $\bar{x} \pm s$. Comparisons of experimental groups were evaluated by Student's t-test; $P < 0.05$ was considered significant.

2 Results

2.1 Protection against lethal virus challenge in mice with plasmids pC4H5 and pCMVH5

The anesthetized mice were challenged with $10^{5.8}$ ELD_{50} H5N1 virus, a lethal dose. Immunization with 30 μg eukaryotic expressing plasmids administered by electroporation, either pC4H5 or pCMVH5, provided 100% protection against death from challenge with H5N1 virus in the complete absence of detectable virus in the lung tissues, while the control groups which were immunized with blank plasmid all died completely after virus challenge (Table 1). Immunization with 30 μg of pC4H5 or pCMVH5 by intramuscular injection also provided 100% protection against death from homologous challenge with H5N1 virus, but could not effectively inhibit to shed viruses in cloaca after challenge.

Table 1 Protection induced by HA-DNA immunization against H5 avian influenza viruses in mice[1)]

Vaccine[2)]	No. mice with viruses replication in lung/No. tested			No. sick/No. dead/total	Protection rate/%
	5 d postinfection	7 d postinfection	16 d postinfection		
pC4H5(E.P.)	0/3[3)]	0/3	0/3	0/0/6	100
pC4H5(I.M.)	3/3	2/3	0/3	6/0/6	100
pCMVH5(E.P.)	0/3	0/3	0/3	0/0/6	100
pCMVH5(I.M.)	3/3	1/3	0/3	6/0/6	100
Blank p. (E.P.)	3/3	3/3	6/6[4)]	6/6/6	0
Blank p. (I.M.)	3/3	3/3	6/6[4)]	6/6/6	0

[1)]The mice were challenged with $10^{5.8}$ ELD_{50} A/Goose/GuangDong/1/96 (H5N1) avian influenza virus by intranasal inoculation.[2)]All mice were immunized with 30 μg plasmids respectively. [3)]The replication of virus in mouse lung was detected by SPF embryonated eggs. [4)]The blank plasmid control mice all died on day 8 or day 9 after challenge.

In addition, all immunized mice by I.M. postinfection on day 5 could detect living viruses in lung, and showed signs of infection with losing 8.3% of body weight. Subsequently, the immunized mice had gradually cleared out the replicated viruses as indicated by disappearance of the transient signs of those of infected mice, and became healthy. Whereas the control mice showed several signs of infection (huddling, shivering, and ruffled fur), and lost up to 23% of their body weight. The survival mice were always monitored for a total of 21 days and were all healthy.

2.2 Protection against lethal virus challenge in chickens with plasmids pC4H5 and pCMVH5

The chickens immunized in 1.3 and 1.4 were challenged with $10^{4.2}$ ELD_{50} H5N1 viruses by intramuscular injection, a lethal dose to chicken. Hundred percent of the chickens immunized with pC4H5 or pCMVH5 by electroporation were protected from challenge with homologous virus without signs of infection (Table 2). And the viruses were inhibited to shed in cloaca when challenge with H5N1. While the chickens immunized by intramuscular injection and the control chickens showed signs of severe infection and 100% mortality by day 5.

Table 2 Protection induced by HA-DNA vaccine immunization against H5 avian influenza viruses in chickens[1)]

Vaccine[2)]	No. positive/ No. tested[3)]	Recovery rate /%	No. sick/ total	No. dead/ total	Protection rate /%
pC4H5 (I.M.)	–[4)]	–	6/6	6/6	0
pC4H5 (E.P.)	0/6	0	0/6	0/6	100
pCMVH5 (I.M.)	–	–	6/6	6/6	0
pCMVH5 (E.P.)	0/6	0	0/6	0/6	100
Blank p. (I.M.)	–	–	6/6	6/6	0
Blank p. (E.P.)	–	–	6/6	6/6	0

[1)]Challenge by intramuscular injection with $10^{4.2}$ ELD_{50} H5N1 viruses. [2)]All chickens were immunized with 50 μg plasmids respectively. [3)]The viruses in cloaca were detected by SPF embryonated eggs. [4)]The immunized chickens were all dead.

2.3 Antibody responses of mice immunized with HA DNA vaccine

The sera of mice were collected every week from the first week. After the treatment with receptor-destroying enzyme, HI titers were determined by hemagglutinin inhibition assay[23,24]. As shown in Figure 1, the groups that were immunized by electroporation produced antibodies from the second week, the antibody levels were enhanced after boosting; while other groups that were injected intramuscularly only produced antibodies from boosting. The antibody titers of immunized mice by eletroporation were obviously higher than those of immunized mice by intramuscular injection. No significant difference was observed between the experimental and control groups before immunization ($P > 0.05$). But when challenge, the two groups showed difference significantly ($P < 0.05$) (Figure 1). The results indicated that the specific antibodies were elicited in the experimental mice after immunization with HA DNA vaccines.

2.4 HI titer detection in immunized chickens

The sera of chickens were collected every week from the first week. HI titers were determined by HI assay. Figure 2 shows that the results were similar with those of immunized mice. The immunized group by eletroporation produced detectable antibodies from the second week. While, the immunized groups by I.M. produced detectable antibodies from the fourth week. The level of HI specific antibodies from E.P. groups is obviously higher than that from I.M. groups. No significant difference was observed between the

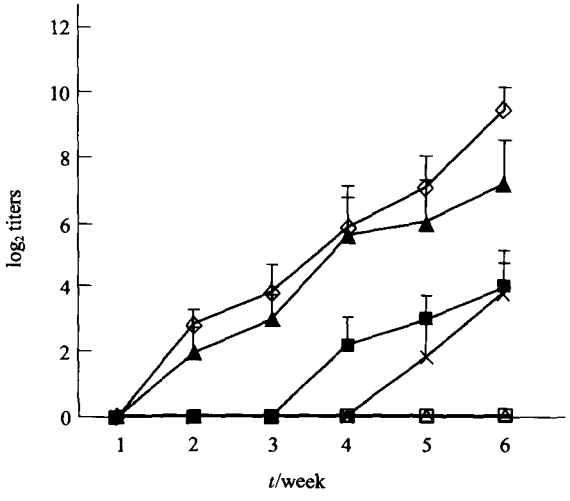

Fig.1 Antibody responses in immunized mice by I.M. and electroporation administration of HA-encoding DNA *in vivo*

Mice were injected with I.M. 30 μg of the plasmid DNA encoding the HA from A/Goose/GuangDong/1/96 (H5N1) influenza virus with or without electroporation twice, 3 weeks apart. Two weeks after the second immunization, the mice were challenged with a lethal dose of $10^{5.8}$ ELD_{50} of HPAIV A/Goose/GuangDong/1/96 (H5N1). The HI titers of different immunized mice in different weeks were measured respectively by hemagglutinin inhibition assays. Each value represents the $\bar{x} \pm s$ in each group of five mice. ◇—◇: pC4H5 (E.P.); ■—■: pC4H5(I.M.); ▲—▲: pCMVH5(E.P.); ×—×: pCMVH5(I.M.); △—△: blank p.(E.P.); □—□: Blank p.(I.M.).

experimental and control groups before immunization ($P > 0.05$), but the significant difference between the two groups was observed ($P < 0.05$) when challenge. The results indicated that the specific antibodies were elicited in the experimental chickens after immunization with HA DNA vaccines.

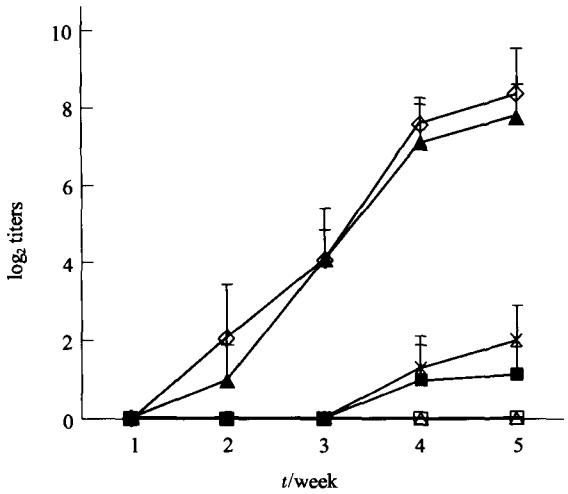

Fig.2 Development of HI antibodies in chicken sera after vaccination with HA DNA vaccines

The experiment group was injected with I.M. 50 μg of the plasmid DNA encoding the HA from A/Goose/GuangDong/1/96 (H5N1) influenza virus with or without electroporation twice, and the control group was injected with blank plasmids. Two weeks after the second immunization, the chickens were challenged with a lethal dose of $10^{4.2}$ ELD_{50} of HPAIV A/Goose/GuangDong/1/96 (H5N1). Serum samples for all chickens were collected each week. Antibody titers were determined with HI assay. The results indicated that the recombinant plasmids could induce an immune response in chicken. Each value represents the $\bar{x}\pm s$ in each group of six chickens. ◇—◇: pC4H5(E.P.); ■—■: pC4H5(I.M.); ▲—▲: pCMVH5(E.P.); ×—×: pCMVH5(I.M.); △—△: Blank p.(E.P.); □—□:Blank p.(I.M.).

2.5 CTL responses were examined after primary and secondary immune responses, compared with different immune methods

The CTL responses were examined in some of the immunized mice described in section **1.7**. During postinfection on day 5, spleen cells from the immunized mice were cultured with inactivated H5N1 virus for 5 days. For controls, spleen cells from mice immunized with blank plasmid were used. Figure 3 shows that spleen cells from immunized mice with expression plasmids exhibited CTL responses that were stronger than those with blank plasmids. And the cytotoxicity of immunized mice with pC4H5 by electroporation was up to 79% (effect : target= 100:1). The significant difference between the experimental and control groups was observed ($P < 0.05$) in the CTL assays. Thus, mice immunized by I.M. administration of 30 μg of HA-DNA with electroporation, twice, 3 weeks apart, induced a relatively strong CTL response.

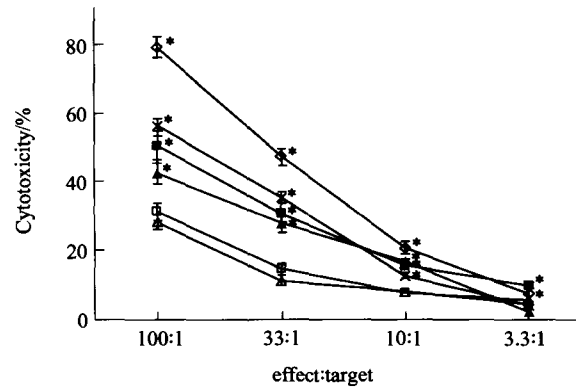

Fig.3 Cytotoxic T-lymphocyte (CTL) activity in immunized mice by either E.P. or I.M. with HA-encoding pC4H5 or pCMVH5

The mice were immunized twice by E.P. and I.M., 3 weeks apart. On the 5th day after challenge, spleen cells from immunized mice were used to detect the CTL activity. Strong responses were observed. Each point represents the $\bar{x}\pm s$ of triplicate samples from pooled spleen cells in each group of three mice. The asterisk (*) denotes statistically significant difference ($P < 0.05$). ◇—◇ :pC4H5 (E.P.); ■—■ :pC4H5 (I.M.); ▲—▲:pCMVH5(E.P.); ×—×:pCMVH5(I.M.); △—△: Blank p.(E.P.); □—□: Blank p.(I.M.).

3 Discussion

An effective influenza DNA vaccine should be developed by determination of the most protective viral protein-expressing DNA and then be testified to have the best protection efficiency against influenza virus. Especially cell-mediated immunity plays an important role in protecting body from virus[25,26]. In the present experiment of mice, the mice were immunized with 30 μg of plasmids (pC4H5 or pCMVH5) DNA by electroporation and were infected lethal doses of H5N1 AIV could obtain 100 percent of protection rate, and then completely inhibited viruses to replicate in their lungs (Table 1). In the previous study, gene gun immunization of 12 mice with 1 μg of pHKHA provided 100% protection against death from homologous challenge with HK97 virus, and one of four mice tested had virus replication in the lungs[4]. While in Kadowaki's experiment, the mice were immunized with 30 μg of pCAGGSP7/HA plasmids by eletroporation and were challenged with lethal doses of PR8 virus only obtained 80% of protection rate[16]. One of the reasons resulted in differences in

these studies most probably lays in different promoters, such as pC4H5, pCMVH5, and pHKHA were controlled by human cytomegalovirus (CMV) immediate- early promoter, while pCAGGSP7/HA was controlled by chicken β-actin promoter. It has been testified that the CMV immediate-early promoter was superior for the induction of immune response by DNA immunization in the mouse model [27]. Another reason probably lays in the better electroporation conditions that each four pulses of both obverse and opposite polarities of 100 V were delivered to each injection site at the rate of one pulse per second, each pulse lasting for 40 ms, as tested previously [22]. In the experiment of chickens, the chichens were completely protected from AIV, and effectively inhibited viruses to shed in chicken cloaca (Table 2). While in Kadihalli's DNA vaccine experiment for chickens, the mice were immunized by gene gun with 10 μg pCMVH5HA and challenged with a lethal dose of Ty/Ir/83 (H5) viruses. They obtained 86% protection rate without shedding viruses in the tested chickens [23]. Comparing the results of mice and chickens in present experiment with those of previous experiments [4,16,27], which suggests that DNA vaccines, pC4H5 and pCMVH5, with electroporation immunization can effectively protect mice and chickens against death from AIV and completely inhibit viruses to replicating after challenge with a lethal dose of A/Goose/GuangDong/1/96 (H5N1) influenza virus.

Antibody production is usually the major mechanism of protection against influenza virus infection after immunization. Antibodies to the HA molecule are necessary if the influenza virus is to be neutralized and the infection is to be prevented [28]. Thus, both mice and chickens by elctroporation immunization that produced higher level of specific antibodies (Figure 1 and 2) could not detect a detectable replicated virus in mouse lungs and chicken cloacas (Table 1 and 2), whereas the mice and chickens immunized by I.M. with low level of specific antibodies were found have replicated virus. The absence of virus replication suggested effective virus neutralization in mice and chickens with DNA encoding HA of H5N1 viruses. The mice immunized by I.M. with a low HI specific antibody level (Figure 1) only from booster recovered from infection of virus. This observation suggests that B-cell memory plays an important role in mediating the immune response to influenza virus [28,29]. Consistent with this conclusion, in previous study that DNA vaccine encoding H5 HA induced protection in mice with low HI antibodies level after booster [23]. Thus, H5-specific memory B cells were activated after challenge infection may have prevented the development of lethal pneumonia following respiratory challenge. *In vivo* electroporation for IL-5 gene transfer could augment the serum IL-5 production more than 100 times of its production by I.M., as suggested by Aihara and Miyazaki, by increasing the number of muscle fibers that took up plasmid DNA, or probably the copy number of plasmids introduced into each muscle cell [15]. In present experiments, it was shown that HA-DNA was transferred into muscle by electroporation induced about 10 times stronger HI antibody responses than HA-DNA administration into muscle without electroporation after challenge (Figure 1 and 2). This result was similar with that of Kadowaki [16], implied *in vivo* eletroporation could reinforce the immune responses elicited by saline HA-DNA administration into muscle.

In our CTL assays, our BALB/c female mice immunized with a dose of 30 μg DNA vaccine, pC4H5 or pCMVH5, by electroporation and challenged with a lethal dose of A/Goose/GuangDong/1/96 (H5N1) virus induced CTL responses with HI specific antibody responses. Another BALB/c mice immunized with pCAGGSP7/HA of DNA vaccine by electroporation and infected with a lethal dose of PR8 virus also induced similarly CTL responses with strong anti-HA Ab responses [16]. $CD8^+$ CTL have been implicated as playing a role in recovery or clearance of virus during a viral infection [8]. The mice immunized by I.M. were detected detectable virus replication and observed signs of infection (Table 1) with a comparatively strong CTL response (Figure 3) and a low HI specific antibody level (Figure 1) on day 5 postinfection. Then, the signs of these infected mice gradually disappeared, and the I.M. immunized mice recovered from infection without replicating viruses in lung. The results indicated that cross-reactive $CD8^+$ CTL and the neutralizing antibody response may both play a part in recovery from a virulent influenza virus infection. Overall, the above conclusions suggest that electroporation is one of the efficient gene delivery systems for the transfer of influenza DNA vaccine in both humoral immunity and cellular immunity.

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用电穿孔方法研究 H5N1 禽流感病毒 DNA 疫苗对动物的免疫保护作用

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摘要 为了研究 H5N1 DNA 疫苗对小鼠和鸡的保护效率，用 H5N1 禽流感病毒 HA DNA 疫苗免疫 BALB/c 小鼠和 SPF 鸡. 小鼠和鸡分别经电穿孔和肌肉注射免疫两次，间隔为 3 周. 二次免疫后，用致死量的同源病毒进行攻毒实验. 空白对照组在攻毒后全部死亡，而经电穿孔免疫的小鼠和鸡均获得了完全的保护，并能有效地抑制病毒在小鼠肺脏和鸡泄殖腔的繁殖. 同时，电穿孔免疫的小鼠和鸡均产生了高水平的特异性抗体. 经电穿孔免疫的小鼠攻毒后 CTL 反应明显加强. 这些结果表明，HA DNA疫苗能有效地保护小鼠和鸡对禽流感病毒的感染，同时也表明电穿孔免疫是 DNA 疫苗免疫的有效途径之一.

关键词 H5N1 禽流感病毒，DNA 疫苗，电穿孔免疫
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