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RESEARCH ARTICLE

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Effect of cell mediated immunity regulation of duck enhanced by duck IFN-α eukaryon expression plasmid and inoculated with DPV attenuated vaccine by gene-gun

Abstract In order to study the effect of cell mediated immunity regulation of duck IFN-α eukaryon expression plasmid (pcDNA-SDIFN-α) on duck plague virus (DPV) attenuated vaccine in ducks, pcDNA-SDIFN-α was administered to 28-day-old ducks at doses of 1, 3 and 6 μg per duck, respectively, by gene-gun. PBS and empty vector pcDNA were used as control. Fifteen days later, all ducks were injected with DPV attenuated vaccine and blood samples were collected at 3, 7, 14, 21, 28, 35, 49, 63 and 84 days after injection. T-lymphocyte proliferation tests (MTT) were used to detect the T-lymphocyte proliferation in the peripheral blood (PBL) of ducks. Blood samples collected at 7, 14, 21, 28, 35 and 49 days after injection were detected by fluorescence-activated cell sorter (FACS) for recording the number of CD_3^+ T-lymphocytes of ducks. Results were as follows: (1) Reaction of T-lymphocytes in PBL to *Con*A (OD value) of ducks treated with pcDNA-SDIFN-α was higher than that of PBS and pcDNA control groups in 3–84 days. There were highly significant differences between the 1 μg per duck group and the two control groups in 3–84 days ($P \leqslant 0.01$), between the 3 μg per duck group and the two control groups in 3–84 days ($P \leqslant 0.01$, $P \leqslant 0.05$), and between the 6 μg per duck group and the two control groups in 7–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$). The significant difference was also present between the groups of 1, 3 and 6 μg per duck in 3–35 days ($P \leqslant 0.05$). However, there was no significant difference between the 3 and 6 μg per duck groups ($P \geqslant 0.05$). The pcDNA control group was higher than PBS control group, but no difference was detected ($P \geqslant 0.05$). (2) Change of the number of CD_3^+ T-lymphocytes in ducks administered with different doses of pcDNA-SDIFN-α was higher than that of PBS and pcDNA control groups in 7–49 days. The change in the 1 μg per duck group was significantly higher than that in PBS and pcDNA control groups in 14–49 days ($P \leqslant 0.01$). There were significant differences between the 3 μg per duck group and the two control groups in 21–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$) and between the 6 μg per duck group and the two control groups in 7–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$). However, no significant differences among the groups of 1, 3, and 6 μg per duck groups ($P \geqslant 0.05$) and between the two control groups ($P \geqslant 0.05$) were found. The results indicated that pcDNA-SDIFN-α administered 15 days before injection of DPV-attenuated vaccine could significantly enhance cellular immunity induced by DPV-attenuated vaccine. pcDNA-SDIFN-α is an excellent DPV-attenuated vaccine molecular adjuvant and the best result can be obtained with the dose of 1 μg per duck of pcDNA-SDIFN-α inoculated by gene-gun.

Keywords gene-gun, duck IFN-α eukaryon expression plasmid, DPV attenuated vaccine, cell mediated immunity, molecular adjuvant

1 Introduction

In the research of the avian flu virus's disturbance phenomenon, Isaacs and Lindanmann (1957) discovered interferon (IFN) in the chicken allantocherion with the function of disturbing virus reproduction. It is a 20–34 ku protein (Lampson et al., 1963). Mammalian interferons can be divided into Type I and Type II interferons. Type I interferon includes IFN-alpha, IFN-beta, IFN-omega and IFN-tau, which can regulate the MHC expression, enhance the macrophage activity, adjust the B cell activity and enhance the cytotoxicity of natural killer cells (NK) and lymphokine-activated killer cells

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(LAK). Type II interferon only contains IFN-gamma, produced by the activated T-lymphocyte induced by an inducer. It can regulate the immune system. Compared with mammalian interferon, research progress of poultry interferon is very slow, with only IFN-alpha and IFN-gamma being discovered. In regulating the expression of MHC Class I antigen, poultry reorganization Type I and Type II interferons play immune functions consistently in mammals (Weining et al., 1996). Some researches reported Duck IFN-alpha (DuIFN-α) could protect duck cells *in vitro* from virus challenge (such as flu virus, vesicular stomatitis virus and new castle disease virus) by cytopathic effect inhibition (CPEI) and could inhibit the reproduction of DHBV on hepatocytes of duck embryo (Schuhz et al., 1995). Eukaryotic expression plasmid DNA vaccine is also known as gene vaccine (DNA vaccine). At present, no influence of the duck IFN-alpha eukaryotic expression vector (pcDNA-SDIFN-α) on the duck cellular immunity at post-inoculated with duck's attenuated vaccine has been reported. This study was based on the previous work in our laboratory (Chen et al., 2006) using pcDNA-SDIFN-α to inoculate 28-day-old ducks at different doses by gene-gun and to inoculate the ducks with DPV-attenuated vaccine15 days later. T-lymphocyte proliferated test (MTT) was used to detect the T-lymphocyte proliferation in the peripheral blood (PBL), fluorescence activated cell sorter (FACS) was used to record $CD3^+$ T-lymphocytes number of dynamic changes in ducks and indirect ELISA was used to measure anti-DPV antibody so as to evaluate the effect of pcDNA-SDIFN-α as an immune modulator and provide tentative data for clinical practices.

2 Materials and methods

2.1 Plasmid and vaccine

Vector pcDNA3.1(+) was purchased from Invitrogen (USA). The pcDNA-SDIFN-α was constructed and prepared in our laboratory. *E. coli* DH5 alpha was conserved in our laboratory and used for pcDNA- SDIFN-α amplification. Immune plasmids were diluted with PBS. DPV attenuated vaccine was supplied by China Animal Husbandry Industry Co. Ltd.

2.2 Tested animals

28-day-old Peking ducks were obtained from the Waterfowl Centre of Sichuan Agricultural University and the specific antibody of DPV was negative. The ducks were arranged into 5 groups with 3 in each group. Group A: pcDNA-SDIFN-α 1 μg per duck; Group B: pcDNA-SDIFN-α 3 μg per duck; Group C: pcDNA-SDIFN-α 6 μg per duck; Group D (attenuated group): PBS 200 μL per duck; Group E (empty vector group): empty vector 100 μg per duck. When the gene gun (DPS 1000/He System, BIO-RAD) bombarded pcDNA-SDIFN-α, the helium pressure was 300 psi (620.55 kPa). Fifteen days later, all ducks were injected with DPV-attenuated vaccine (one immune dose per duck).

2.3 Experimental design

Blood samples were collected at 3, 7, 14, 21, 28, 35, 49, 63, 84 and 105 days and T-lymphocyte proliferation test (MTT) was used to detect the proliferation of ducks T-lymphocytes in the peripheral blood (PBL). Fluorescence-activated cell sorter (FACS) was used to detect the number of CD_3^+ T-lymphocytes of the obtained blood samples at 7, 14, 21, 28, 35 and 49 days after injection.

2.4 Separation of duck PBMCs from whole blood

Blood was collected from the jugular vein of ducks into heparin sodium or EDTA and 1 mL of this diluted blood was layered over 1 mL of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 1500 $r \cdot min^{-1}$ for 20 min at room temperature (RT) as described by Spector et al. (1998). Cells were washed three times in PBS before use.

2.5 T-lymphocyte proliferation assays

PBMCs were cultured at 5×10^6 $cells \cdot mL^{-1}$ with 20 $\mu g \cdot mL^{-1}$ of Concanavalin A (*Con*A, Sigma) in RPMI-1640 medium with 10% FBS, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin and poured into 96 well trays (100 μL per well) for 68 h. After being cultured for 68 h at 37°C in CO_2 incubator (Forma Series II, Thermo), 5 $mg \cdot mL^{-1}$ MTT (10 μL per well, Sigma) was added before the end of cultivation. It was continuously cultivated for 4 h and thereafter, 100 μL of dimethyl sulfoxide (10% SDS-0.04 $mol \cdot L^{-1}$ HCl) was added in each well, mixed completely to dissolve the purple crystal. The plate was then kept at 37°C for 4 h. The optical density was measured at 490 nm on an ELISA Reader (ELx800, Bio-tek).

2.6 Immunofluorescence staining

The procedure was based on the experiment (Edward et al., 1996). Single cell suspensions (5×10^6 cells) of duck lymphoid cells were permeable to antibodies after treatment at 4°C in a buffer containing 45% acetone, 9.25% paraformaldehyde in PBS and were vortexed for 10 sec, followed by a wash in cold PBS + 0.1% bovine serum albumin (BSA) + 0.1% sodium azide (P-B-A) three times. Cells were then incubated with mouse anti-human CD_3^+ (Bio Legend) for 45 min and washed in PBS and 10000 cells

were assayed by Fluorescence-activated cell sorter (Becton-Dickinson).

2.7 Statistical analysis

All statistical analyses were performed using the software SPSS 12.0 for Windows. A two-tailed Student's *t* test was used to compare the differences in mean values between groups. A *P* values less than 0.05 were deemed statistically significant.

3 Results

3.1 Reaction of T-lymphocytes in PBL to *Con*A

After being inoculated with different doses of pcDNA-SDIFN-α, the ducks were injected with DPV-attenuated vaccine. The OD values increased 3 days later, reaching the peak on the 28th day and then fell off (Fig. 1). The reaction of T-lymphocytes in PBL to *Con*A of ducks inoculated with pcDNA-SDIFN-α was higher than that of attenuated vaccine and empty vector control groups in 3–84 days. There was a highly significant difference between the 1 μg per duck group and the two control groups in 3–84 days ($P \leqslant 0.01$). Significant differences were found between the 3 μg per duck group and the two control groups in 3–84 days ($P \leqslant 0.01$, $P \leqslant 0.05$), between the 6 μg per duck group and the two control groups in 7–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$) and between the 1 μg per duck group and the 3.6 μg per duck group in 3–35 days ($P \leqslant 0.05$). However, there was no significant difference between the 3 μg per duck group and the 6 μg per duck group ($P \geqslant 0.05$). The empty vector control group was higher than that of attenuated control group, but no difference was found ($P \geqslant 0.05$).

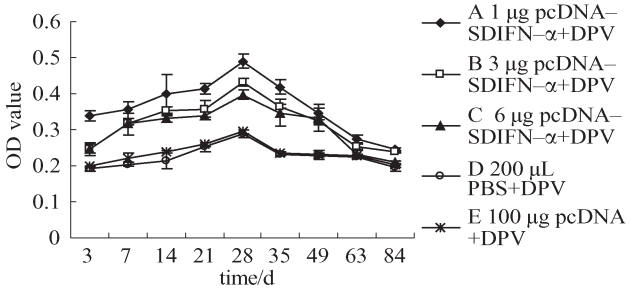

Fig. 1 Proliferation of T-lymphocytes from peripheral blood leukocytes (PBL) in different groups

3.2 Change of the number of CD_3^+ T-lymphocyte

After being inoculated with pcDNA-SDIFN-α at different doses, ducks were injected with DPV-attenuated vaccine, the number of CD_3^+ T-lymphocyte increased after 7 days (Fig. 2). The numbers in the ducks inoculated with different doses of pcDNA-SDIFN-α were higher than that of attenuated and empty vector control groups in 7–49 days. The numbers of the 1 μg per duck group were significantly higher than that of the attenuated vaccine and empty vector control groups in 14–49 days ($P \leqslant 0.01$). There were significant differences between the 3 μg per duck group and the two control groups in 21–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$), and between the 6 μg per duck group and the two control groups in 7–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$). However, no significant difference was found among the 1, 3, and 6 μg per duck groups ($P \geqslant 0.05$) and between the two control groups ($P \geqslant 0.05$).

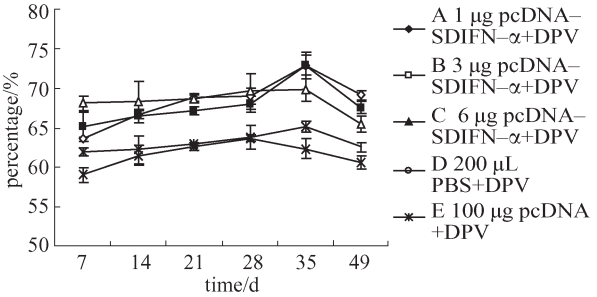

Fig. 2 The dynamic quantity changes of CD_3^+ T-lymphocyte in different groups

4 Discussion

4.1 Function of IFN-alpha molecular immunoadjuvant

IFN can stimulate the immune system indirectly to display its antiviral and immunomodulatory effects. When regulating MHC expression, IFN-α mainly increases MHC Class I expression and enhances cytolytic T-lymphocyte (CTL)-mediated cytotoxicity to promote the cell-mediated effective phase of immune response. IFN-α can also inhibit MHC Class II expression, block MHC Class II restricted helper T-lymphocytes (T_H) and inhibit the identification phase of immune responses. Besides, IFN-α can affect the differentiation and function of dendritic cells (DC) (Luft et al., 1998; Radvanyi et al., 1999; Santini et al., 2000; Tamir et al., 2005), efficiently enhance the activity of macrophages and the function of antigen-presenting cells, mediate T_H cells differentiation to T_{H1} cells, and enhance immune response by secreting cytokines such as IFN-gamma (Tompkins, 1999). IFN-α regulates the immune response through the cytokine network. It enhances the immune response at low dosage, while it inhibits the response at high dosage. Enrico et al. (2002) proved that IFN-α is necessary for the T_{H1} type of immune response induced by typical adjuvants in mice and that IFN itself is an unexpectedly powerful adjuvant when inoculated with the human influenza vaccine for inducing IgG2a and IgA production and providing

protection from virus challenge. Jin et al. (2004) found that the Acquired Immunodeficiency Syndrome Virus core protein (gag) and Interferon (IFNα-2b) fusion genes expression product as an immunogen which could consolidate humoral immunity and can induce mice to produce cellular immunity and respond to HIV-1gag positive serum, indicating that IFN-α is an excellent vaccine molecular adjuvant.

As IFN has the anti-viral function, the inoculation of IFN will also affect attenuated virus reproduction (Zhao et al., 2005). However, the published researches show no difference between exogenous and endogenous IFN-α. Theoretically speaking, DPV-attenuated vaccine can induce IFN-α in ducks. However, some researches indicate that DPV-attenuated vaccine can not affect DPV reproduction at a certain degree and can be retained for a period of time in ducks (Cheng et al., 2005). The expression of pcDNA-SDIFN-α in ducks and IFN-alpha induced spontaneously are the same, but there is still no research reported on duck IFN-alpha molecular immunoadjuvant. Our preliminary test results show that pcDNA-SDIFN-α inoculated with the gene gun can significantly enhance cellular immunity induced by DPV-attenuated vaccine.

4.2 Effect of T-lymphocytes proliferation

It is important to study the cellular function of the efficiency of T-lymphocytes stimulated with mitogen *Con*A transforming into lymphoblast (Roitt et al., 2001). Guo used bovine IFN-α and DNA Vaccine of Foot-and-Mouth Disease Virus to vaccinate Guinea pigs. The result indicated that IFN-α could enhance the effect of T-lymphocytes proliferation (Guo et al., 2004). In our study, we used pcDNA-SDIFN-α to inoculate 28-day-old ducks at different doses by gene gun. Fifteen days later all ducks were injected with DPV- attenuated vaccine and thereafter, 3 days later, the efficiency of T-lymphocytes proliferation in groups treated at different doses was higher than that of the control groups treated with attenuated and empty vector. The results show highly significant differences between the 1 μg per duck group and the two control groups in 3–84 days ($P \leqslant 0.01$), significant differences between the 3 μg per duck group and the two control groups in 3–84 days ($P \leqslant 0.01$, $P \leqslant 0.05$) and between of the 6 μg per duck group and the two control groups in 7–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$), which indicates that pcDNA-SDIFN-α can significantly enhance T-lymphocyte proliferation and increase the cellular immunity induced by DPV-attenuated vaccine. Moreover, ducks inoculated with empty vector (pcDNA) and DPV-attenuated vaccine have more intense responses to *Con*A of PBLs than those inoculated only with attenuated vaccine. The reason is that the plasmid containing a non-CpG methylation at the core of the immune stimulatory sequences (ISS) can enhance non-specific immune response. This also proves that the plasmid itself has immunity adjuvant function by inducing cytokine secretion (Krieg et al., 1998).

4.3 Effect of CD_3^+ lymphocyte from PBL

T-lymphocyte is not only the cellular immunity effector cell, but also the immunoregulation cell. In the immune response, the surface receptors of cytotoxic T cells (Tc), helper T cells (T_H) and suppressor T cells (Ts) can only recognize the antigen fragment of MHC Class I and Class II molecules to generate immune response when forming a complex with surface antigen CD_3^+ (Chen et al., 1995). CD_3^+ is an important marker of T-lymphocytes identification to reflect the number of T-lymphocytes. If it is reduced, the immune response priming, induction and effect may also be reduced. Human CD_3^+ T-lymphocyte and duck CD_3^+ T-lymphocyte have the same antigen. Thus, the anti-human CD_3^+ T-lymphocyte monoclonal antibody may also recognize the duck CD_3^+ T-lymphocyte (Bertram et al., 1996). This is confirmed in our study. In Fig. 2, it can be found that 7 days after different doses of pcDNA-SDIFN-α are inoculated in ducks with DPV-attenuated vaccine, the number of T-lymphocytes in PBL may increase, which is significantly higher in the 1 μg per duck group than that in attenuated and empty vector control groups in 14–49 days ($P \leqslant 0.01$), showing significant differences between the 3 μg per duck group and the two control groups in 21–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$) and between the 6 μg per duck group and the two control groups in 7–49 days ($P \leqslant 0.01$, $P \leqslant 0.05$). Our conclusion is that as DPV-attenuated vaccine regulator, pcDNA-SDIFN-α, can significantly increase CD_3^+ T-lymphocytes from duck BPL and improve the level of the cellular immune responses.

4.4 Effect of DPV attenuated vaccine immune response by immune route, dose and time of pcDNA-SDIFN-α

Using gene-gun to bombard cells with DNA-coated gold microspheres (Tang et al., 1992; Fynan et al., 1993) is the most efficient method to induct DNA vaccine to cells. Compared with intramuscular injection (IM), gene-gun bombardment can reduce DNA vaccine degradation by nuclease *in vivo* to avoid the loss of DNA vaccine. The skin tissue on the inoculation spot contains various types of antigen-presenting cells (APC) and abundant expression of MHC molecules T_H cells which can produce a variety of cytokines and induce the activation of the immune system. These characteristics can guarantee that the gene vaccine can be highly expressed while muscle cells have low level expression of MHC Class II cells and inferior antigen-presenting ability to skin cells (Kang et al., 2004). In this study, groups treated with different doses of pcDNA-SDIFN-alpha could significantly enhance T-lymphocyte proliferation, increase the number of CD_3^+ T-lymphocytes and enhance the level of antibody in the serum. This indicates that gene gun immunization is a

highly effective immunity method with the best dose at 1 μg of pcDNA-SDIFN-α per duck, followed by 3 μg per duck and with 6 μg per duck being the worst.

Animal age has some influences on the gene vaccine expression (Wells and Goldspink, 1992). The 1–28-day-old ducks are at the rapid enhancement stage of cellular immunity levels (Zhang et al., 1990; Cauchy and Malkinson, 1981). To prevent their own cellular immunity system in the development from surmounting and covering the observation of cellular immunoregulation by pcDNA-SDIFN-α, we selected 28-day-old ducks. After the injections to the animals, gene vaccine needs a period of time to express protein function. For example, Chattergoon injected mice with pcENV plasmid to express HIV-1 gp160 protein and the highest level of protein expression appeared at 5–6 days (Chattergoon et al., 1998). Unlike directly injecting IFN- alpha into the ducks, the ducks need a period of time to express IFN-alpha after being injected with pcDNA-SDIFN-alpha. Injecting pcDNA- SDIFN-α 15 days before injecting DPV-attenuated vaccine can significantly enhance cellular immunity and humoral immunity induced by DPV-attenuated vaccine.

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