COEXPRESSION OF INTERLEUKIN-2 ENHANCES THE IMMUNIZATION EFFECT OF A DNA VACCINE EXPRESSING HERPES SIMPLEX 1 GLYCOPROTEIN D

W.-R. LI[1], B. NIU[2], J.-W. WANG[1], Z.-J. FENG[1], D.-X. WANG[1*]

[1]Department of Neurology, Beijing Friendship Hospital Affiliate of Capital University of Medical Sciences, Beijing 100050, P.R. China; [2]Beijing Medical University United Biomedical Center, Beijing 100083, P.R. China

Received March 6, 2006; accepted October 24, 2006

Summary. – In this study, DNA vaccines consisting of vector IRES-gD expressing Herpes simplex virus 1 (HSV-1) glycoprotein D (gD) and vector IRES-gD-IL-2 coexpressing HSV-1 gD and interleukin-2 (IL-2), respectively, were constructed. After intramuscular inoculation, both vaccines induced in BALB/c mice antibodies as assayed by ELISA and virus neutralization. However, IRES-gD-IL-2 elicited significantly higher levels of IgG (ELISA) and neutralizing antibodies than IRES-gD. Isotyping of sera from mice injected with IRES-gD-IL-2 revealed predominately IgG2a antibodies. IRES-gD-IL-2 also elicited a higher delayed-type sensitivity (DTH) reaction. However, there was no difference in the protection against lethal challenge with HSV-1 between the two vaccines ($P>0.05$). The results suggest that the vaccination with IRES-gD-IL-2 can efficiently enhance the immune response of mice to HSV-1, particularly through increased cellular immunity.

Key words: Herpes simplex virus 1; glycoprotein D; interleukin-2; DNA vaccine

Introduction

HSV-1 is responsible for many cases of virus encephalitis, oral herpes and corneal disease in adults (Kennedy, 1988; Cinque *et al.*, 1996). In addition, opportunistic infections with HSV-1 may also occur in immunocompromised patients. Recently, there is growing evidence that several infective agents inclusive of HSV-1 causing persistent or latent chronic infections are possible etiologic cofactors in the pathogenesis of atherosclerosis (Kotronias, 2005; Ibrahim, 2005). Apparently, the development of effective HSV-1 vaccine as the means of specific prevention and even therapy has a great significance (Nesburn *et al.*, 1998; Stanberry *et al.*, 2002). As DNA vaccines produce antigens, which have conformation and antigenicity similar to those of natural antigens, they can induce a broad immune response. Moreover, DNA vaccines are regarded as safe, reliable and efficient. Consequently, they have now become highlights of HSV-1 vaccine research (Ulmer *et al*., 1998; Cafaro *et al.*, 2001; Shata *et al*., 2001).

Among 12 known HSV-1 envelope glycoproteins, gD has a rather strong immunogenicity and can induce high-titer neutralizing antibodies, thus protecting the immunized mice against lethal HSV-1 challenge through antibody-dependent complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (Ghiasi *et al*., 1995). gD has been considered the major candidate immunogen in HSV-1 vaccine research (Stanbery *et al.*, 2002).

Several studies have shown that a co-administration of a vector expressing cytokine can enhance the immune responses of some DNA vaccines (Kim *et al*., 2001; Xin *et al.*, 1998; Ghiasi *et al*., 1995). IL-2 is a kind of cytokine that has wide range of biological effects. It can strengthen humoral and especially cellular immunity (Osorio *et al*., 2003).

Based on these observations, in this study, we constructed DNA vaccines either expressing HSV-1 gD alone or co-

*E-mail: drwania@yahoo.com.cn; fax: +8610-63171389.

Abbreviations: HSV-1 = Herpes simplex virus 1; IL-2 = interleukin-2; gD = glycoprotein D; DTH = delayed-type hypersensitivity; ADCC = antibody-dependent cell-mediated cytotoxicity

expressing HSV-1 gD and IL-2 and investigated their immunogenicity in mice.

Materials and Methods

Virus. HSV-1 strain F was grown in Vero cells in Eagle's Minimal Essential Medium supplemented with 200 mmol/l L-glutamine. 10% of FCS, and antibiotics at 37°C in a humidified atmosphere of 5% CO_2. The virus titer (PFU/ml) was determined by standard plaque assay in Vero cells. Human monocyte-derived dendritic cells were obtained from Dr. B. Niu, Beijing Medical University United Biomedical Center, Beijing, P.R. China.

Vector constructs. The plasmid IRES-gD expressing HSV-1 gD was constructed as follows.

HSV-1 gD gene was amplified by PCR from total DNA isolated from HSV-1-infected Vero cells using the primers 5'-*attata*CTCGAGATGGGGGGGGCTGCC-3' (forward) and 5'-*TCA*CGCGTCTAGTAAAACAAGGGCTG-3' (reverse) complemented with *Xho*I and *Mlu* restriction sites, respectively (the inserted protective nucleotides are in italics). The primers were designed and synthesized by Beijing SaiBaiSheng Genetech Co., Ltd, Beijing, P.R. China. The PCR consisted of denaturation at 94°C for 8 mins, followed by 30 cycles of 94°C/60 secs, 57°C/60 secs, and 72°C/90 secs, and final extension at 72°C for 7 mins. The PCR product was cloned into pCDNAII vector and confirmed by sequencing. The gD gene was finally recloned into the *Xho*I/*Mlu* sites of an expression vector IRES. The resulting vector IRES-gD was identified by restriction analysis and PCR.

The IRES-gD-IL-2 vector expressing human IL-2 was constructed as follows. The IL-2 gene was amplified by PCR from pET-3a/DT_{389}-IL-2 vector (obtained from Dr. J.-W. Wang, Chinese Center for Disease Control and Prevention, Beijing, P.R. China) using the primers 5'-*GCC*GTCGACATGTACAGGATGCAACTCCT GTCTTGCATTGCACTAAGTCTTGCA-3' (forward) and 5'-*GTA*GCGGCCGCTCAAGTCAGTGTTGAGAT-3' (reverse) complemented with *Sal*I and *Not*I restriction sites, respectively (the inserted protective nucleotides are in italics). The primers were designed and synthesized by Beijing SaiBaiSheng Genetech Co., Ltd, Beijing, P.R. China. The PCR consisted of denaturation at 94°C for 4 mins, followed by 30 cycles of 94°C/45 secs, 51°C/45 secs, and 72°C/60 secs, and final extension at 72°C for 7 mins. The PCR product was cloned into the *Sal*I/*Not*I sites of IRES expression vector. The resulting vector IRES-IL-2 was recloned into the corresponding sites of IRES-gD vector to produce finally IRES-gD-IL-2. The latter was confirmed by restriction analysis and PCR. Large amounts of recombinant vectors were prepared using a Qiagen Column (Qiagen) and endotoxin-free buffers.

Animals and immunization. Six-week-old male BALB/c mice purchased from Institute of Experimental Animals, Chinese Academy of Medical Sciences, Beijing, P.R. China, were used. Groups of 10 mice were injected with 100 µl of a DNA vaccine in Tris-borate buffer (1 µg DNA/µl) into the quadriceps muscles of both hind legs. Fifteen mins before the immunization, 100 µl of 25% sucrose was injected in the same way to enhance DNA vaccine absorption. The mice were boosted with the same dose of vaccine 2 and 4 weeks later. Before immunization and one week after each immunization, blood samples were taken from the tail vein. The mice immunized with original IRES vector served as a negative control.

Western blot analysis was performed according to Sanna *et al.* (1995). After SDS-PAGE, the HSV-1 gD was detected with goat anti-HSV-1 gD polyclonal antibody (Santa Cruz Inc., USA), HRP-labeled anti-goat IgG, and the ECL visualization system (Kuo *et al.*, 2002).

ELISA of IgG, IgG1 and IgG2 antibodies to HSV-1 gD. Microplates (Costar) were coated with UV-inactivated HSV-1 F strain diluted in a coating buffer (0.01 mol/l $NaHCO_3$ pH 9.6) overnight at 4°C, washed with PBS-T buffer, and blocked for 2 hrs at 37°C with PBS-T containing 1% of FCS. After washing, serial dilutions of sera were added in duplicate and the plates were incubated for 1 hr at 37°C. After washing, a 1:5,000 dilution of goat HRP-labeled anti-mouse IgG (Santa Cruz Inc., USA), used as secondary antibody, was added and the plates were incubated for 1 hr at 37°C. After washing, an ABTS (Sigma) solution was added for 20 mins and A_{490} was read.

The titer was defined as the reciprocal of the highest dilution of serum, which gave a reading that was at least 2-times higher as compared with a control and exceeded 0.1. For the detection of IgG isotype antibodies, an 1:4,000 dilution of goat HRP-labeled anti-mouse IgG2 or IgG1 (Fisher Scientific Inc.) was employed. A_{490} readings instead of titers were directly employed for comparison of the DNA vaccines as regards individual IgG isotype antibodies.

Titration of neutralizing antibodies to HSV-1 was carried out in 96-well microplates (Nunc). Three IgG-positive sera obtained from the second booster immunization with the DNA vaccines were randomly selected, inactivated at 56°C for 30 mins, diluted in DMEM, incubated with 1,000 PFU of HSV-1 at 37°C for 1 hr, and added to Vero cells (0.2 ml/well) in duplicate. Standard controls were employed. The cultures were kept at 37°C and observed daily for CPE. The antibody titer was defined as a reciprocal of the highest serum dilution that inhibited viral CPE.

DTH assay. One week after the second booster immunization, 6×10^5 PFU of HSV-1 (10 µl) inactivated at 60°C for 1 hr was injected subcutaneously into the right auricle of mice. The same amount of a Vero cell lysate was injected into the left auricle as a control. Two days later, the

thickness of right and left ear was measured with an engineer's micrometer. The DTH response in each mouse was expressed as the thickness difference between both auricles.

Assay of survival of mice after challenge. Two weeks after the second booster immunization, 1×10^6 PFU of HSV-1 (0.5 ml) was injected intraperitoneally into mice. The survival of mice was observed for 2 weeks.

Statistical analysis. The one-way ANOVA and Fisher's exact test were used to determine significance of differences. The significance was positive for $P>0.05$.

Results

The *in vitro* expression of gD from the vectors IRES-gD and IRES-gD-IL-2, and that of IL-2 from the vectors IRES-gD-IL-2 and IL-2, respectively, was verified by transfection of human dendritic cells with the vectors followed by Western blot analysis. In the lysates of cells transfected with IRES-gD and IRES-gD-IL-2, respectively, a specific band of 45 K corresponding to gD was detected. This indicated that the gD expressed by dendritic cells had a good immunoreactivity. In the lysates of cells transfected with IRES-IL-2 and IRES-gD-IL-2, respectively, a specific band of 15 K corresponding to IL-2 could be seen (data not shown).

To study the induction of IgG antibodies, mice were immunized intramuscularly with IRES-gD and IRES-gD-IL-2, respectively, while the original IRES vector served as control (Fig. 1). The serum IgG HSV-1 antibody titers after the first and two booster doses were determined by ELISA. The antibodies appeared first following the first booster immunization with either vaccine. IRES-gD-IL-2 was a stronger inducer than IRES-gD, which was more apparent following the second booster immunization as compared with the first one.

To further characterize the immune response elicited to HSV-1, the IgG antibody-positive sera were analyzed for their isotype by ELISA (Table 1). The results showed that

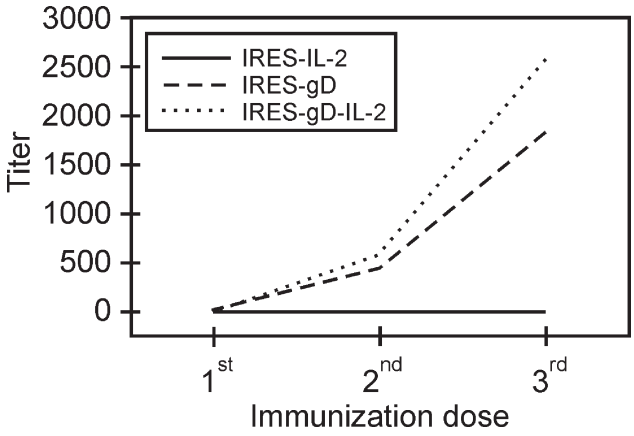

Fig. 1

ELISA titers of IgG antibodies to HSV-1 gD in sera of immunized mice

Mice were bled 2 weeks after the second booster immunization. The mice injected with IRES vector represented a negative control. Data represent averages from 10 serum samples.

the A_{490} readings for both IgG1 and IgG2a antibodies elicited with IRES-gD-IL-2 were significantly higher as compared with IRES-gD. The IgG2a/IgG1 ratio gave a similar picture. These and above results indicated that both cellular and humoral immunities induced with IRES-gD-IL-2 were stronger than those with IRES-gD, and that the Th1 cell response was dominant (Table 1).

Next, the induction of serum HSV-1 neutralizing antibodies in mice after the second booster immunization with the DNA vaccines was examined. It was found that the titers for IRES-gD-IL-2 were higher (64) than those for IRES-gD (32).

In further experiments, the DTH response of mice to immunization with the DNA vaccines and challenge with inactivated virus was investigated. The DTH response to IRES-gD-IL-2 was found to be stronger than that to IRES-gD (Table 2).

Table 1. Induction of IgG1 and IgG2a antibodies to HSV-1 with DNA vaccines in mice

	ELISA A_{490}		
	IgG1	IgG2a	IgG2a/IgG1
Control	0.068±0.004	0.076±0.003	1.12
IRES-IL-2	0.069±0.009	0.084±0.012	1.22
IRES-gD	0.342±0.085*	0.976±0.077*	2.85
IRES-gD-IL-2	0.673±0.100*	2.987±0.524*	4.44

The sera obtained from mice 2 weeks after the second booster immunization. Values differing significantly from that of control are marked with asterisk (*).

Table 2. DTH response to challenge in mice immunized with DNA vaccines

	DTH response (μm)
Control	14.8±1.6
IRES-IL-2	19.8±5.8
IRES-gD	120.8±24.6*
IRES-gD-IL-2	132.6±45.7**

DTH response represents the difference in the thickness of left and right auricle in μm. Data are the means±SD from groups of 10 mice. Statistically significant differences ($P<0.05$) are indicated by single asterisk (*) when compared with negative control, and by double asterisk (**) when compared with IRES-gD.

Table 3. Survival of mice immunized with DNA vaccines after challenge

	No. of survived/dead mice	Survival rate (%)
Control	0/8	0
IRES-IL-2	1/10	10
IRES-gD	7/10	70
IRES-gD-IL-2	6/8	75

The mice challenged with the virus 2 weeks after the second booster immunization.

To prove that IL-2 can increase protection against lethal challenge with HSV-1 in mice immunized with HSV-1 gD, mice were immunized with IRES-gD and IRES-gD-IL2, respectively, and were challenged with HSV-1 two weeks after the second booster. Non-immunized mice and those immunized with IRES-IL-2 served as controls (Table 3). The results showed that while all the 10 control mice and 9 of 10 mice given IRES-IL-2 died, 7 of 10 mice given IRES-gD and 6 of 8 mice given IRES-gD-IL-2 survived. Hence these two vaccines obviously protected the majority of challenged mice from lethal viral effect. However, the difference between the two vaccines was not significant.

Discussion

Many studies have proved that among 12 HSV-1 glycoproteins, gD has strongest immunogenicity, induces high titer antibodies to HSV-1 and protects animals against lethal viral challenge (Ghiasi *et al.*, 1995; Dean *et al.*, 1994; Martin, 1987). Cytokines are a group of heterogeneous peptide regulatory factors. Since they can regulate the cells related to the immunity of the body, they play an important role in innate and adaptive immunity (Chow *et al.*, 1998; Kim *et al.*, 2000). It has been shown that cytokine gene adjuvants (GM-CSF, IL-12, and IL-12) can better enhance immunization effects than traditional ones (Barouch *et al.*, 1998). It is presumed that a cytokine gene adjuvant expresses cytokine and thus stimulates immunity-related cells and promotes humoral and cellular immunities for a long time. Recent reports have shown that IL-2 expressed from the respective gene adjuvant induced stronger humoral and cell-mediated immunity (Chow *et al.*, 1997; Xin *et al.*, 1998; Kim *et al.*, 1999, 2001). Since virus replication occurs inside the cells, humoral immunity plays a limited role in HSV-1 infection. IL-2 is a crucial cytokine in cellular immunity, which suggests the important function of IL-2 in HSV-1 infection.

Based on these observations, we constructed IRES-gD, a vector expressing HSV-1 gD, and IRES-gD-IL-2, a vector expressing both HSV-1 gD and human IL-2. Immunoblot analysis showed that these vectors expressed *in vitro* the respective proteins. As a part of this analysis was based on specific binding of the HSV-1 gD antibody to HSV-1 gD, it proved the presence of a specific epitope on the expressed HSV-1 gD and indicated that the constructed DNA vaccines carrying the HSV-1 gD gene would have a rather good immunogenic activity. The immunization of mice with these two kinds of DNA vaccines resulted in a specific HSV-1 antibody response. After two booster immunizations, the average IgG antibody titer for IRES-gD-IL-2 (2,600) was significantly higher than that for IRES-gD (1,800). The highest individual titers were 6,400 and 3,200, respectively.

As it is neutralizing antibodies that block the binding of the virus to its receptor(s) on the surface of susceptible cells, thus making the virus lose its infection ability or neuroinvasiveness, this kind of antibodies play an important role in preventing HSV-1 infection. Previous studies have proved that vaccination with a purified HSV-1 gD can induce high-titer neutralizing antibodies to HSV-1 (Straus *et al.*, 1993; Ghiasi, 1991). Other works have shown that neutralizing antibodies alone could provide a sufficient protection against lethal HSV-1 challenge (Bourne *et al.*, 1996). In this study, we showed that the vaccination with IRES-gD-IL-2 induced neutralizing antibodies with an average titer of 64, which was significantly higher as compared with IRES-gD (32).

Besides humoral also cell-mediated immunity plays an important role in the protection against HSV-1 infection (Larsen *et al.*, 1983; Rouse *et al.*, 1994). Th1 cells secrete IL-2 and IFN-γ to mediate cellular immune response; Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 to function in the generation of antibodies, in humoral immunity (Mosmann *et al*., 1989; Paul *et al*., 1994). By secreting IL-4 and IL-5, Th2 cells promote B cells to generate high-titer IgG1, IgA and IgE antibodies. Th1 cells inhibit the synthesis of IgG1, but promote the synthesis of IgG2 antibodies. Therefore, if the level of IgG1 is higher than that of IgG2a, the Th2 response dominates and promotes humoral immunity. On the contrary, if the level of IgG2a is higher than that of IgG1, the Th1 response dominates and mediates cellular immunity, since Th1-secreted cytokines promote the activation of Tc cells, and IgG2a itself activates complement and binds Fc receptor on the cellular surface to activate antibody-dependent cell-mediated cytotoxicity (Yang *et al*., 2001).

In comparing the isotype profiles of the antibody response and DTH reaction to measure the dominance of immune response, we observed that the levels of IgG1 and IgG2a with IRES-gD-IL-2 were significantly higher than those with IRES-gD, indicating that both humoral and cellular immunities were stronger with IRES-gD-IL-2 than those with IRES-gD. In addition, the IgG2a/IgG1 ratio with IRES-gD-IL-2 was greater than 1, indicating that the Th1 response dominated. Especially IgG2a was enhanced more markedly, causing the increase in IgG2a/IgG1. The results of DTH

response also indicated that it was stronger with IRES-gD-IL-2 as compared with IRES-gD. The IgG2a/IgG1 ratios and DTH responses demonstrated in accord with previous reports that the co-expression of IL-2 enhanced the cellular immune response to DNA vaccine expressing HSV-1 gD.

To understand the protective effect of the DNA vaccines against lethal HSV-1 challenge, the mice immunized with the DNA vaccines were challenged with the virus. The results showed that the survival rates of mice immunized with the DNA vaccines increased over the control very markedly but so similarly that the effects of the two vaccines could not be distinguished. This indicates that although an IL-2 gene adjuvant can induce a stronger cellular immune response and more protective antibodies than HSV-1 gD DNA vaccine alone, total amount of antibodies is still rather low to neutralize the lethal virus dose completely and thus to augment significantly the protective effect of HSV-1 gD DNA vaccine. Improvement of the immunogenicity of IRES-gD-IL-2 is objective of further research.

Acknowledgements. The authors thank Drs. J. Wang, Q. Yang and L. Yang from the Institute of Virus Control and Prevention, and the United Biomedical Center, University of Beijing, respectively, for great help and valuable discussion throughout the course of the investigation.

References

Barouch DH, Santra S, Steenbeke TD, Zheng XX, Perry HC, Davies ME, Freed DC, Craiu A, Strom TB, Shiver JW, Letvin NL (1998): Augmentation and suppression of immune responses to a HIV DNA vaccine by plasmid cytokine/ Ig administration. *J. Immunol.* **161**, 1875–1882.

Bourne N, Stanberry LR, Bernstein D, Lew D (1996): DNA immunization against experimental genital herpes simplex virus infection. *J. Infect. Dis.* **173**, 800–807.

Cafaro A, Titti F, Fracasso C, Maggiorella MT, Baroncelli S, Caputo A, Goletti D, Borsetti A, Pace M, Fanales-Belasio E, Ridolfi B, Negri DR, Sernicola L, Belli R, Corrias F, Macchia I, Leone P, Michelini Z, ten Haaft P, Butto S, Verani P, Ensoli B (2001): Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). *Vaccine* **19**, 2862–2877.

Caselli E, Grandi P, Argnani R, Balboni PG, Selvatici R, Manservigi R (2001): Mice genetic immunization with plasmid DNA encoding a secreted form of HSV-1 gB induces a protective immune response against herpes simplex virus type 1 infection. *Intervirology* **44**, 1–7.

Chow YH, Huang WL, Cli WK, Chu YD, Tao MH (1997): Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin 2. *J. Virol.* **71**, 169–178.

Chow YH, Chiang BL, Lee YL, Chi WK, Lin WC, Chen YT, Tao MH (1998): Development of Th1 and Th2 populations and the nature of immune responses to hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine genes. *J. Immunol.* **160**, 1320–1329.

Cinque P, Clertor GM, Weber T, Monteyne P, Sindic CJ, van Loon AM (1996): The role of laboratory investigation in the diagnosis and management of patients with suspected herpes simplex encephalitis: a consensus report. *J. Neurol. Neurosurg. Psychiatry* **61**, 339–345.

Dean HJ, Terhune SS, Shieh MT, Susmarski N, Spear PG (1994): Single amino acid substitution in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alteration of infectivity. *Virology* **199**, 67–80.

Ghebrekidan H, Ruden U, Cox S, Wahren B, Grandien M (1999): Prevalence of herpes simplex virus types 1 and 2, cytomegalovirus, and varicella-zoster virus infections in Eritrea. *J. Clin. Virol.* **12**, 53–64.

Ghiasi H, Kaiwar R, Nesburn AB, Kaiwar R, Wechsler SL (1991): Immunoselection of recombinant baculoviruses expressing high levels of biologically active herpes simplex virus type 1 glycoprotein D. *Arch. Virol.* **121**, 163–178.

Ghiasi H, Bahri S, Nesburn AB, Wechsler SL (1995): Protection against herpes simplex virus-induced eye disease after vaccination with seven individually expressed herpes simplex virus 1 glycoproteins. *Invest. Ophthalmol. Vis. Sci.* **36**, 1352–1360.

Ibrahim AI, Obeid MT, Jouma MJ, Moasis GA, Al-Richane WL, Kindermann I, Boehm M, Roemer K, Mueller-Lantzsch N, Gartner BC (2005): Detection of herpes simplex virus, cytomegalovirus and Epstein-Barr virus DNA in atherosclerotic plaques and in unaffected bypass grafts. *J. Clin. Virol.* **32**, 29–32.

Kennedy PGE (1988): A retrospective analysis of forty-six cases of herpes simplex encephalitis seen in Glasgow between 1962 and 1985. *Q. J. Med.* **255**, 533–540.

Kim JJ, Simbiri KA, Sin JI, Dang K, Oh J, Dentchev T, Lee D, Nottingham LK, Chalian AA, McCallus D, Ciccarelli R, Agadjanyan MG, Weiner DB (1999): Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J. Interferon Cytokine Res.* **19**, 77–84.

Kim JJ, Yang JS, Dentchev T, Dang K, Weiner DB (2000): Chemokine gene adjuvants can modulate immune responses induced by DNA vaccines. *J. Interferon Cytokine Res.* **20**, 487–498.

Kim JJ, Yang JS, Manson KH, Weiner DB (2001): Modulation of antigen-specific cellular immune responses to DNA vaccine in rhesus macaques through the use of IL-2, IFN-gamma , or IL-4 gene adjuvant. *Vaccine* **19**, 2496–2505.

Kotronias D, Kapranos N (2005): Herpes simplex virus as a determinant risk factor for coronary artery atherosclerosis and myocardial infarction. *In Vivo* **19**, 351–357.

Larsen HS, Russell RG, Rouse BT (1983): Recovery from lethal herpes simplex virus type 1 infection is mediated by cytotoxic T lymphocytes. *Infect. Immun.* **41**, 197–204.

Martin S, Moss B, Berman PW, Laskey LA, Rouse BT (1987): Mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus 1 glycoprotein D: cytotoxic T cell. *J. Virol.* **61**, 726–734.

Mosmann TR, Coffman RL (1989): TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**, 145–173.

Nesburn AB, Burke RL, Ghiasi H, Slanina SM, Wechsler SL (1998): A therapeutic vaccine that reduces recurrent herpes simplex virus type 1 corneal disease. *Invest. Ophthalmol. Vis. Sci.* **39**, 1163–1170.

Osorio Y, Sharifi BG, Perng GC, Ghiasi NS, Ghiasi H (2002): The role of TH1 and TH2 cytokines in HSV-1-induced corneal scarring. *Ocular Immunol. Inflammation* **10**, 105–116.

Osorio Y, Ghiasi V (2003): Comparison of adjuvant efficacy of herpes simplex virus type 1 recombinant viruses expressing TH1 and TH2 cytokine genes. *J. Virol.* **77**, 5774-5783.

Paul WE, Seder RA (1994): Lymphocyte responses and cytokines. *Cell* **76**, 241–251.

Sanna PP, Williamson RA, De Logu A, Bloom FE, Burton DR (1995): Directed selection of recombinant human monoclonal antibodies to herpes simplex virus glycoproteins from phage display libraries. *Proc. Natl. Acad. Sci. USA* **92**, 6439–6443.

Rouse RJ, Nair SK, Lydy SL, Bowen JC, Rouse BT (1994): Induction in vitro of primary cytotoxic T-lymphocyte responses with DNA encoding herpes simplex virus proteins. *J. Virol.* **68**, 5685–5689.

Shata MT, Hone DM (2001): Vaccination with a shigella DNA vaccine vector induces antigen-specific CD8(+) t cells and antiviral protective immunity. *J. Virol.* **75**, 9665–9670.

Stanbery LR, Spruance SL, Cunningham AL, Bernstein DI, Mindel A, Sacks S, Tyring S, Aoki FY, Slaoui M, Denis M, Vandepapeliere P, Dubin G; Glaxo Smith Kline Herpes Vaccine Efficacy Study Group (2002): Glycoprotein D adjuvant vaccine to prevent genital herpes. *N. Engl. J. Med.* **347**, 1652–1661.

Straus SE, Savarese B, Tigges M, Freifeld AG, Krause PR, Margolis DM, Meier JL, Paar DP, Adair SF, Dina D (1993): Induction and enhancement of immune responses to herpes simplex virus type 2 in humans by use of a recombinant glycoprotein D vaccine. *J. Infect. Dis.* **343**, 1460–1463.

Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, Liu X, Wang S, Liu MA, Donnelly JJ, Caulfield MJ (1998): Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. *J. Virol.* **72**, 5648–5653.

Xin KQ, Hamajima K, Sasar S, Fukushima J, Kaneko T, Xin KQ, Kudoh I, Okuda K (1998): Intranasal administration of human immuno-deficiency virus type-1 (HIV-1) DNA vaccine with interleukin-2 expression plasmid enhances cell-mediated immunity against HIV-1. *Immunology* **94**, 438–444.

Yang K, Wang S, Chang KO, Lu S, Saif LJ, Greenberg HB, Brinker JP, Herrmann JE (2001): Immune responses and protection obtained with rotavirus VP6 DNA vaccines given by intramuscular injection. *Vaccine* **19**, 3285–3291.

Kuo YC, Lin LC, Tsai WJ, Chou CJ, Kung SH, Ho YH (2002): Samarangenin B from *Limonium sinenses s*uppresses herpes simplex virus type 1 replication in Vero cells by regulation of viral macromolecular synthesis. *Antimicrob. Agents Chemother*. **46**, 2854–2864.