IMMUNIZATION WITH VARICELLA-ZOSTER VIRUS GLYCOPROTEIN E EXPRESSING VECTORS: COMPARISON OF ANTIBODY RESPONSE TO DNA VACCINE AND RECOMBINANT VACCINIA VIRUS

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Summary. – Immunization with DNA vaccines expressing Varicella-zoster virus (VZV) glycoprotein E (gE) induced formation of specific antibodies in mice. The antibody response correlated with the level of *in vitro* gE expression if the plasmid was inoculated intradermally (i.d.) with a gene gun but not if intramuscular (i.m.) injection was used. The i.d. vaccination produced a higher antibody level than the i.m. one even though a 100-fold amount of DNA was administered. A plasmid expressing a truncated form of gE was less immunogenic. The magnitude of antibody response induced by immunization with recombinant vaccinia viruses (rVVs) was equivalent to the gene gun vaccination. Administration of DNA by i.m. route or Vaccinia virus (VV) gE by i.d. route resulted in predominance of IgG2a in the response while the gene gun plasmid inoculation usually elicited similar levels of IgG1 and IgG2a. The antibody response elicited by DNA vaccine was boosted by a secondary immunization with rVV. The boosting effect was highest if the virus was administered intraperitoneal (i.p.).

Keywords: Varicella-zoster virus; glycoprotein E; DNA vaccine; Vaccinia virus

Introduction

VZV is a double-stranded DNA virus that belongs to the species *Human herpesvirus 3*, genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae.* It is the causative agent of two distinct diseases, varicella (chickenpox), a clinical manifestation of primary infection in children, and herpes zoster (shingles), which occurs predominantly in adults as consequence of reactivation of latent VZV. Varicella has traditionally been considered a benign and relatively mild infection of childhood, but in neonates as well as in immunocompromised subjects of all ages it may induce a serious encephalitis-like illness or pneumonitis, often with a fatal outcome. The host immune response after natural primary or recurrent infection or vaccination with the VZV-Oka vaccine is mainly directed against the viral glycoproteins, which are structural components of the virion envelope. gE (90–98 K) is the most abundant glycoprotein on VZV-infected cell membranes (Dubey *et al.*, 1988; Montalvo and Grose, 1986). The VZV gE is a typical type I transmembrane glycoprotein consisting of three regions: a hydrophilic extracellular domain of 544 amino acids (aa), a hydrophobic transmembrane region of 17 aa, and a charged cytoplasmic tail of 62 aa (Grose, 1990). In the presence of complement, antibodies to gE (gE antibodies) neutralize infectious virus *in vitro*

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Abbreviations: VZV = Varicella-zoster virus; gE = glycoprotein E; gES = soluble form of gE; i.d. = intradermally; i.m. = intramuscular; rVV = recombinant Vaccinia virus; aa = amino acid; APCs = antigen-presenting cells; i.p. = intraperitoneal; VV = Vaccinia virus; HCMV = Human cytomegalovirus; TK = thymidine kinase; HA = hemagglutinin; PBS = phosphate-buffered saline; MAb = monoclonal antibody; ELISA = enzyme-linked immunosorbent assay

(Grose and Litwin, 1988; Ludvikova *et al.*, 1991). VZV also induces T helper cell responses and contains epitopes recognized by CD4+ and CD8+ cells (Garcia-Valcarcel *et al.*, 1997). So far, the only available vaccine affording protection from natural infection is the live attenuated VZV-Oka vaccine. Despite the success of this vaccine one problem still remains: the Oka strain has retained the ability to establish a latent infection in dorsal root ganglia and can induce herpes zoster after reactivation (Hardy *et al.*, 1991). For this reason, search for alternative vaccines continues.

DNA and poxvirus vectors belong to those genetically engineered vectors which come under consideration for trials of vaccine safety and efficacy in humans. These types of vectors have been reported to induce antibodies and T cell responses against herpesviruses and particularly VZV in experimental animals (Hasan *et al.*, 2000; Lowry *et al.*, 1992; Ludvikova *et al.*, 1991; Massaer *et al.*, 1999; Strasser *et al.*, 2000; Sullivan and Smith, 1987; Wachsman *et al.*, 1987).

In this study we compared antibody responses induced by VZV gE as immunogen inserted into vectors of the above two types and administered by different routes. Experimental animals were immunized with plasmid DNA and/or rVV expressing VZV gE. In the case of immunization with naked DNA, we examined several parameters that might influence antibody production, such as the form of antigen, its expression level and the route of plasmid inoculation: i.d. by gene gun versus i.m. injection. Activation of T helper cells that plays a pivotal role in the immune response by promoting both B and cytotoxic T cell responses requires presentation of the antigen by professional antigen-presenting cells (APCs). A DNA vaccine encoding secreted viral protein might be more effective by generating larger quantities of released antigen available for processing and presentation by APCs and in consequence enhancing the resulting immune response (Boyle *et al*., 1997). To test this hypothesis we constructed and compared vectors expressing either a full-length or truncated form of VZV gE. With a rVV used as vaccination vector, we compared i.p. and i.d. administration. Recently, a novel vaccination regimen representing DNA priming followed by boosting with an attenuated poxvirus vector has been reported to be effective in generation of high levels of specific immunity in Human immunodeficiency virus 1, Simian immunodeficiency virus and malaria models, and in some cases it afforded protection against challenge (Allen *et al.*, 2000; Hanke *et al.*, 1998; Kent *et al.*, 1998; Schneider *et al.*, 1998). On the basis of these findings we tested the possible synergism of a DNA prime and rVV boost in VZV immunization.

Materials and Methods

Viruses and cells. VZV strain Zuzana has been isolated in our laboratory from skin vesicles of a child with varicella and propagated in human embryo diploid (LEP) cells. rVVs were prepared from clone P13 or P20, both derived from the Sevac VARIE smallpox vaccine (strain Praha) (Kutinova *et al.*, 1995). The VZV gene 68 encoding gE (Davison and Scott, 1985)was inserted into the VV thymidine kinase (TK) or hemagglutinin (HA) gene as described previously (Kutinova *et al.*, 1999; Ludvikova *et al.*, 1991). Expression of the extrinsic genes was controlled by the early/late p7.5k VV promoter. The VVs used for immunization experiments were grown in chorioallantoic membranes of 11-day-old chicken embryos and partially purified as described previously (Kutinova *et al.*, 1999). CV-1 cell cultures were used for VV propagation. Cell lines 3T3 and 293T were employed in transfection experiments.

Plasmid vectors. Two plasmid vectors were used in the study: pSG5 (Stratagene), in which transcription of the VZV gE gene was driven by the early SV40 promoter, and pBK-CMVΔ containing the immediate early Human cytomegalovirus (HCMV) promoter. The plasmid pBK-CMVΔ was prepared from pBK-CMV (Stratagene) by excision of the *Nhe*I-*Spe*I fragment, which contained the lac promoter. The gene for VZV gE has originally been excised from the plasmid pVZV-*Hind* III-C (Ecker and Hyman, 1982) and inserted into the plasmid pGS20 (Ludvikova *et al.*, 1991). The plasmid pGS20-VZV gE was digested with *Bam*HI and the resulting 2.3 kbp fragment containing the gE gene was subcloned into the *Bam*HI site of both the pSG5 and pBK-CMVΔ plasmids (Fig. 1). To prepare a truncated soluble form of gE (gES) lacking the transmembrane anchor (aa 539–559) and C-terminal domain, the plasmid pBKΔ-gE was digested with *Sgr*I and *Xma*I and recirculated by ligation. The resulting plasmid, denoted pBKΔ-gES, encoded the aa 1–517 of gE (Davison and Scott, 1985). Plasmid vectors without insert were used as controls. The plasmids were amplified in transformed *E. coli* strain XLl-Blue and purified by a cesium chloride gradient centrifugation.

In vitro expression of antigens. Transfection of cells was performed using the FuGENE 6 Transfection Reagent (Boehringer-Mannheim) following the manufacturer's protocol. The expression of the full-length and truncated forms of gE in transfected 3T3 and 293T cells, respectively, was confirmed by Western blot analysis. The cells were harvested 48 hrs after transfection, washed twice with PBS and lysed in the Laemmli buffer containing 2-mercaptoethanol. The samples were heated at 95°C for 5 mins. SDS-PAGE was carried out in 7.5% gel. The separated proteins were electroblotted to nitrocellulose membranes by the semidry method. The membranes, preincubated for 1 hr with 10% skimmed milk in phosphate-buffered saline (PBS), were incubated at 4°C overnight with a gE-specific mouse monoclonal antibody (MAb) (3B3, a kind gift from Prof. C. Grose, University of Iowa, Iowa City), diluted 1:500 in PBS containing 5% skimmed milk. After washing in PBS with 0.2% Tween 5 times for 5 mins, the membranes were incubated for 1 hr with a rabbit anti-mouse IgG, labeled with horseradish peroxidase (Sigma) diluted 1:4000 in PBS with 5% skimmed milk. The membranes were washed again as above, developed with ECL (Amersham) and autoradiographed.

DNA immunization. Four-week-old outbred female mice strain CD-1 (ICR) (Charles River) were inoculated either i.d. by a gene gun (Helios Gene Gun, BioRad) or i.m. by injection. The i.d. inoculation was performed using 1 μg DNA and 0.5 mg of 1.6 μm gold particles per shot. DNA-coated beads were prepared according to the manufacturer's instructions and delivered into sha-

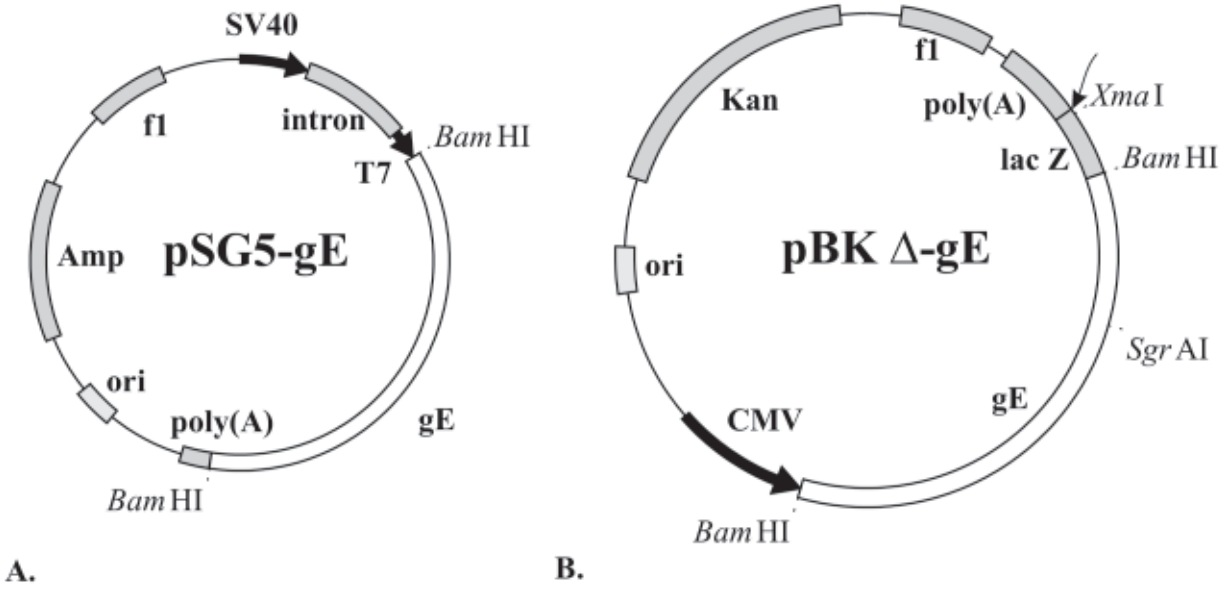


Fig. 1
Expression vectors used in DNA vaccination

Transcription of the gE gene in plasmid pSG5-gE (A) was under the control of the early SV40 promoter. In plasmid pBKDΔ-gE (B) the expression of gE was driven by the HCMV immediate early promoter. The restriction sites *Sgr*I and *Xma*I denote the fragment removed when the gES was generated.

ved abdominal skin using a helium pulse setting of 400 psi. The i.m. injection was done into the *quadriceps femoris* muscle; each injection containing 100 µg DNA diluted in 50 µl of PBS. The immunization scheme was as follows: in the experiment I, mice received equal plasmid doses at time 0 and 8 and 12 weeks later. Before each booster inoculation, the blood was collected from tail veins and the sera of each group of mice were pooled. Sixteen weeks after the first DNA immunization animals were anesthetized with halothane (Léčiva, Praha) and bled out. In the experiment II, the immunization schedule consisted of only two doses given 4 weeks apart. The animals were bled out 8 weeks after the first immunization.

Immunization with rVV. Groups of 16 four-week-old outbred female mice of strain ICR were injected with 10^6 PFU of rVV in 0.5 ml of PBS i.p. or i.d. in the tail base with the virus in 3 µl of PBS. In the prime/boost experiment the animals were injected with rVV at the age of 9 weeks.

Measurement of VZV gE antibodies and VV antigens. Sera of immunized mice were individually tested by enzyme-linked immunosorbent assay (ELISA) for the presence of specific anti-VZVgE antibody. Plates were coated with VZV antigens prepared from LEP cells infected with VZV as described previously (Kutinova *et al.*, 1999; Ludvikova *et al.*, 1991) and incubated with diluted murine sera. A horseradish peroxidase-conjugated swine anti-mouse IgG (Sevapharma) diluted 1:2,000 was used as the detecting antibody and the reaction was visualized with o-phenylene diamine. The level of anti-gE antibody was expressed as the A_{492} value. The antibody isotype was determined in plates coated with the same VZV antigen, using a horseradish peroxidase-conjugated rat anti-mouse IgG2a or IgG1 (Pharmingen) diluted 1:500. The amount of anti-gE specific immunoglobulin isotype is given in units (U), 1 U being defined as the lowest antibody concentration that gives the A_{492} of 0.1 in ELISA. Values were calculated from appropriate dilutions of the sera tested, using one specimen of anti-gE-positive mouse serum as standard for either isotype; it contained 640 U of anti-gE IgG1 and 1280 U of IgG2a. VV-specific antibodies in individual mouse sera were determined by ELISA as described previously (Ludvikova *et al.*, 1991).

Neutralization assay. The VZV-neutralizing capacity of mouse sera was determined by a plaque reduction test. A cell-free virus for the assay was prepared similarly as described (Ilobi and Martin, 1989). In brief, LEP cells infected with VZV strain Zuzana exhibiting approximately 50–70% cytopathic effect were scraped into E-MEM supplemented with 5% of sucrose, 0.1% of sodium glutamate and 10% of heat-inactivated newborn calf serum. The resulting cell suspension was sonicated (Soniprep, MSE) at an amplitude of 14 µm for 30 secs and centrifuged at 950 x g for 15 mins. The supernatant was used as cell-free virus preparation and stored in aliquots at -65°C. Equal volumes of the virus, serial dilutions of heat-inactivated (56°C/30 mins) mouse serum, and a guinea pig serum diluted 1:4 as source of complement, were mixed and incubated at 34°C for 1 hr. In control, mouse serum was replaced by medium. Two Petri dish cultures of LEP cells were inoculated with 0.3 ml of virus and control mixture, respectively. After 30 mins of incubation at room temperature, 5 ml of medium (E-MEM with 10% of heat-inactivated newborn calf serum) was added. Following 10 days of incubation at 37°C, the medium was removed and the cells were stained for at least 1 hr at room temperature with 0.05% neutral red in E-MEM supplemented with 1% of calf serum. Plaques were counted immediately after removal of the stai-

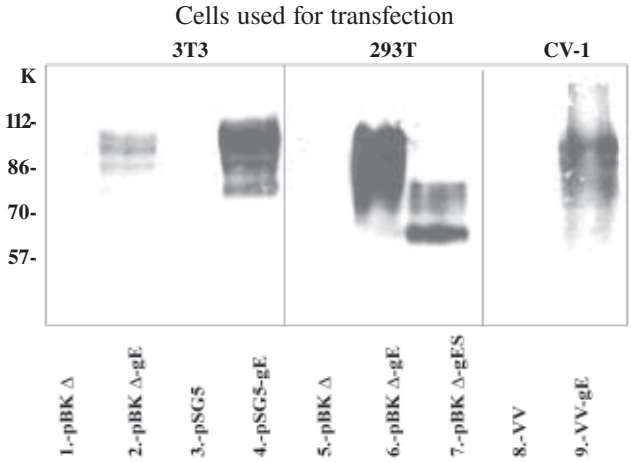


Fig. 2
***In vitro* expression of VZV gE**

Monolayers of 3T3 cells (lanes 1–4) and 293T cells (lanes 5–7) were transfected with plasmid DNA. CV-1 cells (lanes 8–9) were infected with VVs. Glycoproteins were detected by Western blot analysis using the anti-gE MAb.

ning medium. The neutralizing titer was expressed as the reciprocal of the highest serum dilution causing ≥50% plaque count reduction in comparison with control.

Results

In vitro expression of VZV gE

The ability of plasmids pBKΔ-gE, pSG5-gE and pBKΔ-gES to express full-length or truncated VZV gE was demonstrated by transient transfection of 3T3 and 293 T cells. A broad 90–98 K band corresponding to mature glycosylated and various intermediate forms of gE was detected in 3T3 cells transfected with pBKΔ-gE or pSG5-gE by immunoblot analysis using the anti-gE MAb (Fig. 2, lanes 2 and 4). Similar forms of gE molecules were observed in CV-1 cells infected with the VV gE recombinant (lane 9). However, Western blot results showed some differences in efficiency of VZV gE expression. In 3T3 cells, its expression from the pSG5-gE plasmid under control of the SV40 promoter was assessed as at least 3-fold higher than from the pBKΔ-gE plasmid, controlled by the HCMV promoter. Expression of gE lacking the transmembrane domain from the plasmid pBKΔ-gES was demonstrated in transfected 293 T cells: truncated gE molecules ranging from 60 to 70 K (lane 7) were found in the cell lysate 48 hrs after transfection; however, no gE was detected in the culture media (results not shown).

Humoral immune response

In experiment I, groups of eight mice were immunized with one of the plasmids carrying the gE gene. Plasmids without insert served as controls. The results in Fig. 3 indicated that the animals receiving the plasmid pBKΔ-gE or pSG5-gE responded by development of gE antibodies. As expected, inoculation of the control plasmid elicited no gE antibodies. With either of the plasmids, i.d. was superior to i.m. inoculation. The highest level of antibodies was observed in animals inoculated by gene gun with the plasmid pSG5-gE containing the SV40 promoter. Under this arrangement gE antibodies were induced already after one DNA dose; in all other groups the responses to the first dose were very low. Paradoxically, i.m. administration elicited gE antibodies only in animals inoculated with the plasmid pBKΔ-gE carrying the HCMV promoter, but not with the plasmid pSG5-gE. The gene gun inoculation induced antibody responses in all animals in the group, while i.m. immunization with the plasmid pBKΔ-gE resulted in a positive response in only 50% of the animals injected. Immunization by either method produced the highest levels of antibodies at the week 12 in response to the second DNA dose. The third plasmid dose did not further raise the antibody levels, and therefore a later immunization schedule consisted of two DNA doses only.

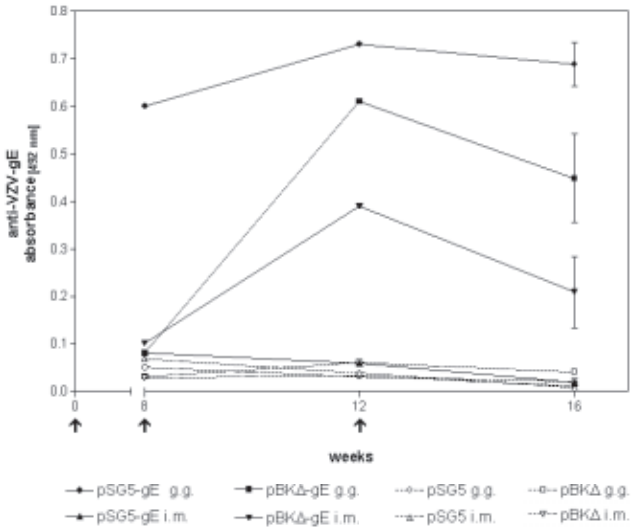


Fig. 3
Antibody response of mice to DNA vaccines encoding the entire gE gene

Mice were immunized with three consecutive plasmid doses administered by gene gun (g.g.) or i.m. at weeks 0, 8 and 12 (arrows). Blood samples taken at weeks 8 and 12 were pooled. Sera obtained at week 16 were assayed individually. Sera were diluted 1/10 and analyzed for total anti-gE immunoglobulins by ELISA. The antibody responses were expressed as A_{492}.

Effect of the gE gene form on antibody induction

In an attempt to improve the antibody responses obtained with full-length gE in the experiment I, the plasmid expressing a truncated form of the gE gene under control of the HCMV

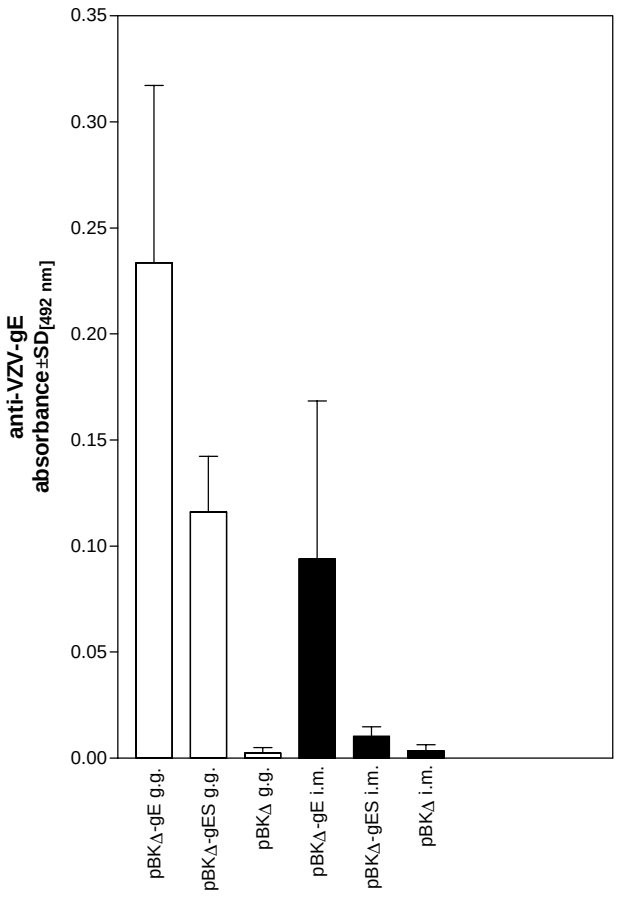


Fig. 4
Antibody response of mice to DNA vaccines encoding a truncated gE gene

Mice were immunized with two plasmid doses 4 weeks apart by gene gun (g.g.) or i.m. Blood samples were taken 8 weeks after the first dose administered. Sera were diluted 1/10 and assayed individually for total anti-gE immunoglobulins by ELISA.

promoter was used for immunization. However, the results (Fig. 4) showed that this plasmid induced lower specific antibody responses than did plasmids containing the complete gE coding sequence. The differences observed between the other plasmids administered by alternative inoculation routes in the experiment II were similar to those seen in the experiment I (Fig. 3).

Virus neutralization activity of gE antibodies induced by DNA vaccines and rVVs

Our next goal was to examine VZV-neutralization activity of the sera of mice immunized with two doses of DNA and compare the antibody responses with those obtained in mice immunized with a single i.p. or i.d. dose of 10^6 PFU of rVV expressing VZV gE. We used two rVVs, generated from the attenuated clone 20 and from the less attenuated clone 13 of the strain Praha. DNA immunization by gene gun induced similar levels of gE antibodies as did the inoculation of one dose of rVV generated from the attenuated clone 20 of the strain Praha; the less attenuated VV clone 13 elicited stronger anti-gE responses (Table 1).

Table 1. VZV neutralization by sera of mice immunized with plasmid DNA or inoculated with rVVs expressing gE

Immunization with[a]	Neutralization titers of sera[b]	VZV gE antibodies[c] (A_{492} ± SD)
PBKD-gE g.g	4	0.280 ± 0.210
PBKΔ g.g	<4	0.001 ± 0.001
PSG5-gE g.g.	16	0.460 ± 0.200
PSG5 g.g.	<4	0.002 ± 0.001
PBKΔ-gE i.m.	4	0.127 ± 0.130
PBKΔ i.m.	<4	0.001 ± 0.001
VV 20-gE-HA i.p.	16	0.470 ± 0.080
VV 20-gE-TK i.p.	8	0.280 ± 0.180
VV 13-gE-TK i.p.	32	0.660 ± 0.270
VV 13-gE-TK i.d.	16	0.490 ± 0.220
VV 13	<4	0.003 ± 0.002

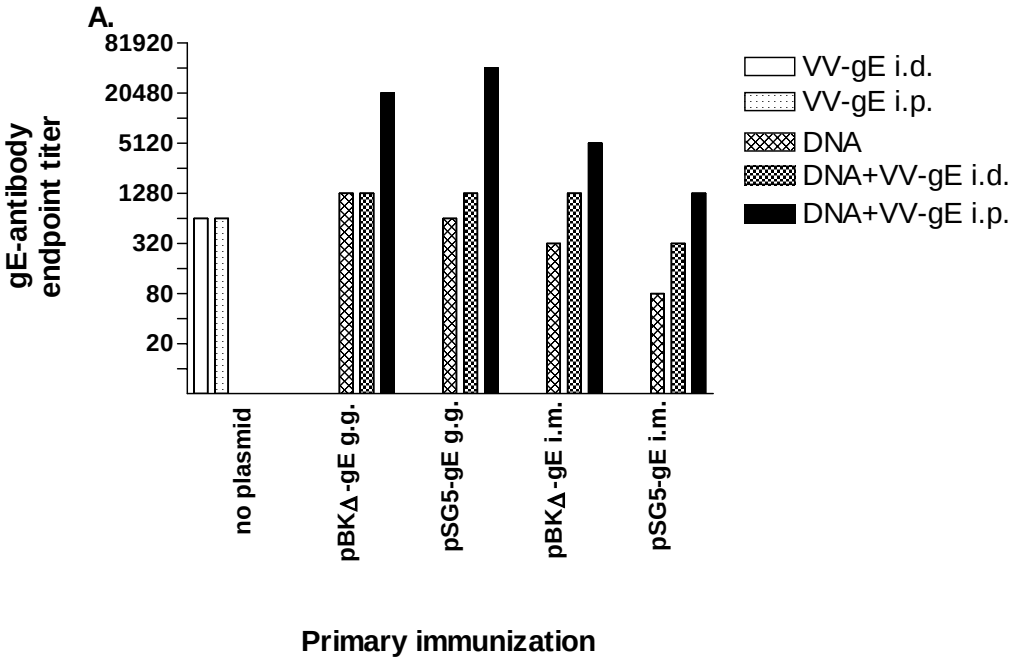
[a]Mice were immunized with two doses of plasmid DNA 4 weeks apart by gene gun (g.g.) or i.m. and blood samples were taken 8 weeks after the first dose administered. Another group of animals was inoculated with 10^6 PFU of VV gE i.p. or i.d. and bled out five weeks later.
[b]Reciprocals of highest serum dilutions that neutralized at least 50% of VZV plaques.
[c]Individual sera of all animals were diluted 1/10 and assayed for total gE antibodies. The values represent arithmetic means ± SD. SD = standard deviation.

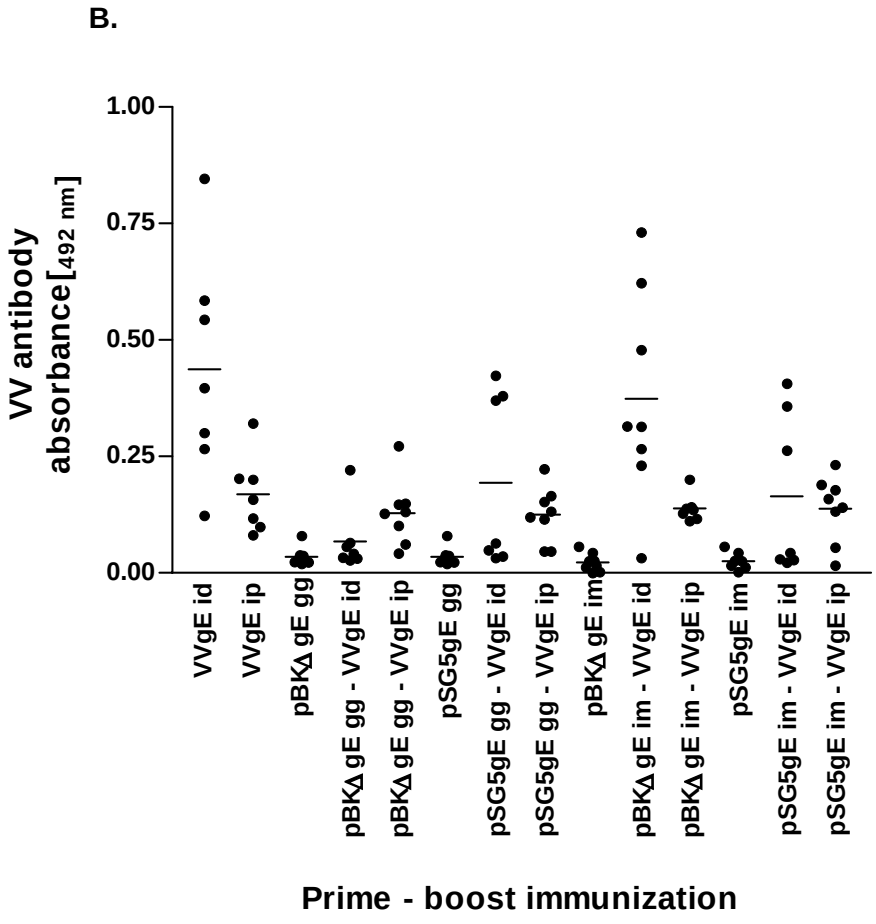
Pooled sera were examined for VZV-neutralizing activity. The capability of these sera to neutralize viral infectivity *in vitro* was evaluated by the plaque reduction assay (Table 1).

After DNA immunization, the highest titers were found in sera of mice inoculated with pSG5-gE by gene gun. Similar levels of neutralizing activity were induced by i.p. inoculation of VV20-gE-HA or by i.d. inoculation of VV13-gE-TK. Immunization with pBKΔ-gE by either route resulted in a lower VZV-neutralizing activity of sera. The administration of empty plasmids or parental VV induced no VZV-neutralizing antibodies.

Boosting of DNA vaccine-induced anti-VZV gE humoral responses with rVV

As the third dose of DNA vaccine was not able to further increase gE antibodies, we were interested in what effect would be produced by rVV, which had proven very effective in boosting immune responses to different antigens. We compared different combinations of administration routes of the prime and boost. Mice were immunized with two doses of the plasmid pBK-gE or pSG5-gE by gene gun or i.m. on days 0 and 14. For the boosting, one dose of 10^6 PFU of recombinant VV13-gE was inoculated i.p. or i.d. on day 35. All animals were bled out on day 64.


A.
81920
20480
5120
1280
320
80
20
gE-antibody endpoint titer
VV-gE i.d.
VV-gE i.p.
DNA
DNA+VV-gE i.d.
DNA+VV-gE i.p.
no plasmid
pBKΔ-gE g.g.
pSG5-gE g.g.
pBKΔ-gE i.m.
pSG5-gE i.m.
Primary immunization


B.
1.00
0.75
0.50
0.25
0.00
VV antibody absorbance[492 nm]
VVgE id
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pBKΔ gE gg
pBKΔ gE gg - VVgE id
pBKΔ gE gg - VVgE ip
pSG5gE gg
pSG5gE gg - VVgE id
pSG5gE gg - VVgE ip
pBKΔ gE im
pBKΔ gE im - VVgE id
pBKΔ gE im - VVgE ip
pSG5gE im
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pSG5gE im - VVgE ip
Prime - boost immunization

Fig. 5

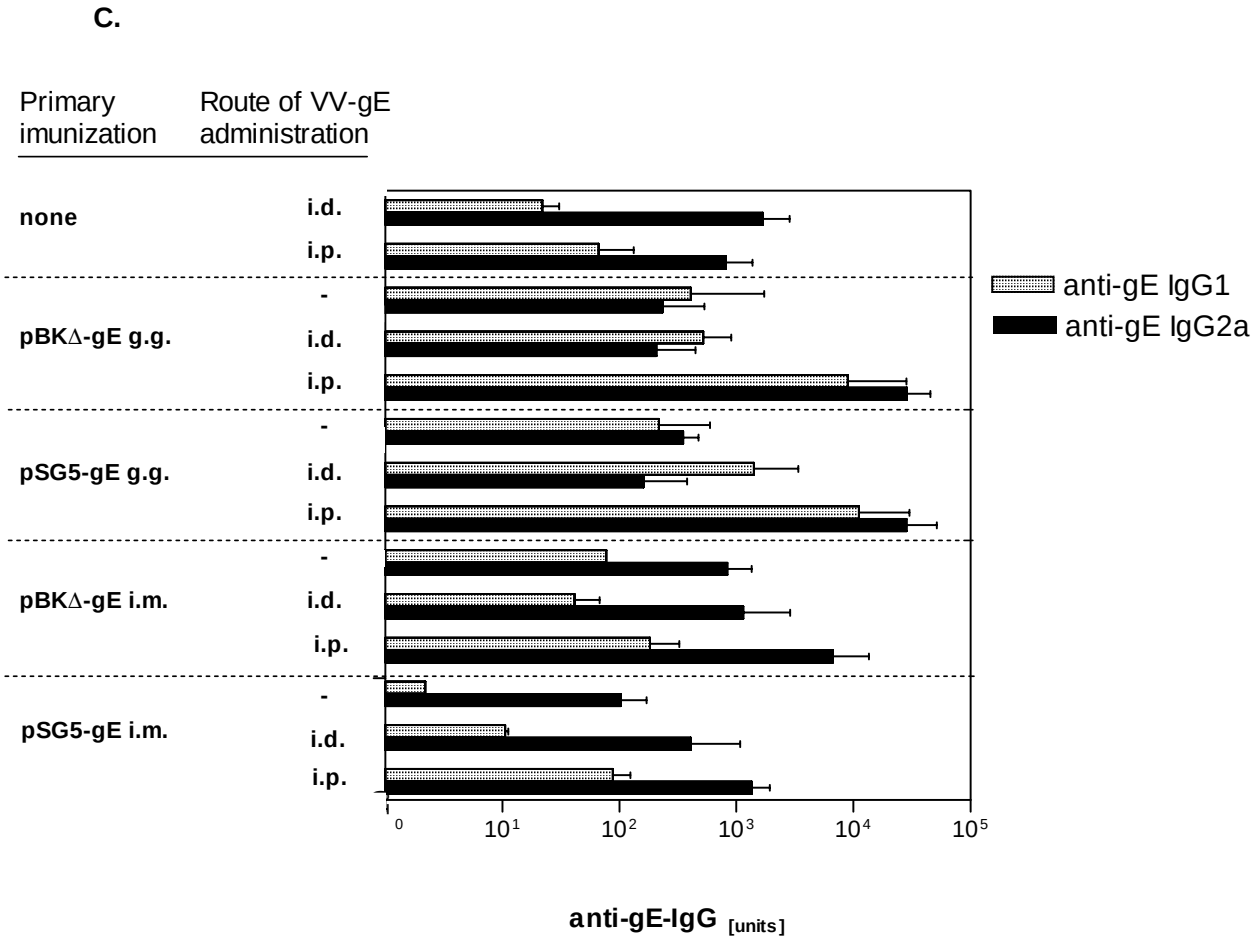

Fig. 5
Prime and boost of gE antibodies in mice

VZV gE-specific antibodies were measured in mouse sera 4 weeks after the last immunization. Serum aliquots were pooled and serial dilutions of sera were examined by ELISA for total gE-specific antibodies. The endpoint titer is the highest reciprocal serum dilution giving A_{492} higher than the mean A_{492} ± 3 SD of sera of mice immunized with the control plasmid diluted 1:10 (A). Individual sera were examined for the presence of VV-specific antibodies by ELISA (B). In positive individual sera, anti-gE specific IgG1 and IgG2a isotypes were determined by ELISA (C).

The endpoint titers of gE antibodies present in pooled mouse sera of each group are shown in Fig. 5A. The DNA immunization by gene gun resulted in the induction of similar levels of gE antibody as did inoculation of one dose of recombinant VV13-gE. The antibody titer after i.m. inoculation of DNA was lower. However, two i.m. injections of pSG5-gE two weeks apart induced a higher anti-gE response than that in previous experiment (Fig. 5A), where the interval between the first and second DNA dose was 8 weeks. VV13-gE virus inoculated i.p. boosted gE antibodies in animals that had received the DNA doses by either route. The boosting effect after i.p. administration of rVV was remarkable if DNA had been administered by gene gun, but it was lower in combination with DNA injected i.m. Surprisingly, there was only very low if any boosting effect after i.d. inoculation of VV. Levels of VV antibodies, which corresponded to VV multiplication were measured in individual sera (Fig. 5 B).

The i.p. inoculated VV elicited similar levels of VV antibodies in all groups of mice irrespective of previous DNA vaccination. Anti-VV responses induced by i.d. inoculated virus were suppressed in many DNA-immunized animals as compared with mice inoculated i.d. with VV13-gE only.

To compare the immune responses in more detail, we determined the isotypes of gE-specific immunoglobulins in individual gE antibodies-positive sera. (Fig. 5C). Similar levels of IgG1 and IgG2a antibodies were produced after immunization with DNA by gene gun, but gE IgG2a prevailed in mice inoculated with DNA administered i.m. The rVV alone, regardless of the administration route, also induced predominantly IgG2a antibodies. VV13-gE given i.p. efficiently

boosted both isotypes in mice primed with DNA by gene gun, but the increase in IgG2a levels was more pronounced. The secondary vaccination with i.p. dose of VV13-gE did not change the predominance of the IgG2a isotype in mice primed with DNA administered i.m. The rVV inoculated i.d. had no significant boosting effect on the isotype profiles.

Discussion

The present study focused on the comparison of quantitative and qualitative differences in humoral immune responses to VZV gE associated with the type of vector and route of administration. We tested two plasmid vectors with different promoters, which induced distinct levels of antigen in cells transfected *in vitro*. A correlation between the levels of *in vitro* antigen expression and antibody induction after i.d. immunization by gene gun was found: higher levels of gE expression from plasmid pSG5-gE as compared to pBKΔ-gE observed in mouse 3T3 cells *in vitro* corresponded to an increased humoral response to the former plasmid. The same plasmids when used for i.m. immunization gave different results. Specific antibodies were detected in animals vaccinated with the plasmid pBKΔ-gE, while the i.m. immunization with pSG5-gE was less effective (Fig. 3). This implies that a high level of *in vitro* expression of gE does not guarantee a potent immune response after i.m. administration of DNA vaccine. It had been observed already previously for a rabies virus glycoprotein that there need not always be a correlation between high antigen expression *in vitro* and immunogenicity *in vivo* (Xiang *et al.*, 1995).

The different efficacy after i.m. and i.d. inoculation observed with either of our plasmids may have been related to the type of cells in the target tissue (muscle vs. skin) and to the way of immunogen administration (i.m. or i.d. injection vs. gene gun). Our results were consistent with a previous observation that a 100-fold DNA dose given by injection generated lower antibody levels than did a normal DNA dose administered by gene gun. Moreover, some of the animals immunized with pBKΔ-gE i.m. did not produce any gE antibodies in the present experiments. The different efficiency of both inoculation methods was apparently caused by different mechanisms of transfection: whereas a gene gun delivers DNA directly into the cells, an injection of DNA in saline by i.d or i.m. route deposits it extracellularly, where its majority is degraded by nucleases (Levy *et al.*, 1996).

gE is a membrane-associated protein, the accumulation of which could be toxic for the cell. Consequently, only limited amounts of this antigen might be presented to the surface of cells transfected *in vivo*. Therefore, a gE encoding gene with the transmembrane region cut out was generated and tested in mice. However, *in vitro* tests showed that the excision of 106 C-terminal amino acids from gE did not result in secretion of detectable amounts of the truncated molecule into the medium of transfected cells. Vaccination of mice with the plasmid pBKΔ-gES expressing this truncated gE resulted in a weaker antibody response than that with plasmids encoding the native form of gE. Similar results have been reported earlier (Massaer *et al.*, 1999). The level of glycosylation and cellular location of full-length and truncated forms of VZV gE have been thoroughly described earlier by Zhu and coworkers (Zhu *et al.*, 1995). As a result of a targeting sequence within the cytosolic domain, gE is accumulated in the *trans*-Golgi network. In contrast, the truncated molecule is retained and degraded in the endoplasmatic reticulum. However, deletion of the transmembrane and cytosolic domains does not affect its N-glycosylation. With reference to these findings we conclude that the lesser immunogenicity of our DNA vaccine delivering gES was due to low amounts of the antigen produced in consequence of a high instability of gES in transfected cells.

It has been previously shown that distinct mechanisms of immune response induction could be triggered when different routes of antigen delivery were employed, and this could influence the isotype profile of the antibodies generated: the injection of DNA in saline into the skin or muscle would predominantly provoke a Th1 response, with the IgG2a antibody prevailing, while inoculation of DNA by gene gun would result in a Th2 response with predominance of IgG1 antibody (Allen *et al.*, 2000; Feltquate *et al.*, 1997; Hanke *et al.*, 1998; Pertmer *et al.,* 1996). Our immunoglobulin isotype analysis of gE antibodies in sera obtained after gene gun immunization demonstrated similar levels of both IgG1 and IgG2a. This differed from the antibody profile of i.m. immunized animals, in which the IgG2a isotype prevailed.

In order to define the efficiency of immunization with VZV gE-expressing plasmids more distinctly, we compared antibody responses elicited by DNA vaccines and by rVVs. The total antibody levels induced by two doses of plasmid DNA administered by gene gun corresponded to the i.p. vaccination with one dose of 10^6 PFU of the recombinant VV-gE derived from the highly attenuated VV20, while the less attenuated VV13 induced higher levels of VZV gE antibodies.

Vaccination with recombinant VVs induced antibodies with a prevalence of the IgG2a isotype. The generation of Thl-biased responses could be ascribed to the effect of interferon g, which is induced in great amounts during virus infection. VZV gE antibodies at the highest IgG2a/IgG1 ratio were elicited by either i.d. vaccination with VV or i.m. immunization with plasmid DNA.

Recombinant poxviruses have been reported to boost both DNA primed $CD8^+$ cell responses (Allen *et al.*, 2000;

Hanke *et al.*, 1998; Schneider *et al.*, 1998) and antibody responses (Fuller *et al.*, 1997). In this paper we report that much higher VZV gE-specific antibody levels were induced by boosting with rVV than with a third dose of the same plasmid DNA. The boosting effect was mainly associated with the inoculation route of virus, because only the VV13-gE administered i.p. boosted gE antibodies, while the virus inoculated i.d. was almost ineffective. The rVV administered i.p. elicited the same levels of VV-specific antibodies in all groups of mice, this implying that all the viruses multiplied similarly irrespective of previous DNA immunization. In contrast, the rVV inoculated i.d. induced lower levels of VV-specific antibodies in some mice pre-immunized with plasmids than in mice which were not subjected to DNA immunization. This might indicate a restricted multiplication of rVV (administered i.d) caused by previous immunity to a transgene. Control of infection and growth of rVV by immunity to an extrinsic antigen such as the nucleoprotein of Vesicular stomatitis virus (Bachmann *et al.*, 1994), a Human immunodeficiency virus 1 peptide (Belyakov *et al.*, 1998) or Human papilloma virus 16 capsid protein (Marais *et al.*, 1999) has been observed repeatedly.

Highest boosts of IgG1 and IgG2a isotypes were obtained in mice primarily immunized with DNA by gene gun in contrast to i.m. immunization. The boost of VZV gE IgG2a was superior in mice primed by gene gun than by i.m. route, even though the initial IgG2a levels induced by i.m. DNA administration were been higher.

From the data presented here we conclude that the highest antibody responses to VZV gE can be induced by a DNA vaccine administered by gene gun in combination with an i.p. boost by rVV.

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