High-Level Cellular and Humoral Immune Responses in Guinea Pigs Immunized Intradermally with a Heat-Inactivated Varicella-Zoster Virus Vaccine

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The threat of varicella and herpes zoster in immunocompromised individuals necessitates the development of a safe and effective varicella-zoster virus (VZV) vaccine. The immune responses of guinea pigs to the intradermal (i.d.) or subcutaneous (s.c.) administration of a heat-inactivated or live VZV vaccine were investigated. Relative to nonimmunized animals, a single 399-PFU dose of vaccine induced nonsignificant increases in gamma interferon (IFN-γ), granzyme B, and perforin mRNA expression in the splenocytes of all groups, while two i.d. administrations of the inactivated vaccine increased IFN-γ mRNA expression significantly ($P < 0.005$). A single 1,995-PFU dose significantly increased the expression of IFN-γ mRNA in the groups receiving the vaccine either i.d. ($P < 0.005$) or s.c. ($P < 0.05$), that of granzyme B mRNA in the groups immunized i.d. with the inactivated ($P < 0.005$) or live ($P < 0.005$) vaccine, and that of perforin mRNA in the animals that received the inactivated vaccine i.d. ($P < 0.005$). Importantly, increases in the expression of IFN-γ ($P = 0.025$), granzyme B ($P = 0.004$), and perforin ($P > 0.05$) mRNAs were observed in the animals immunized i.d. with 1,995 PFU of inactivated vaccine relative to those immunized s.c. with the same dose. The proportion of animals expressing IFN-γ mRNA mirrored the proportion expressing IFN-γ protein (correlation coefficient of 0.88). VZV glycoprotein-specific and virus-neutralizing antibodies were produced with no significant intergroup differences. A booster i.d. administration of the 399-PFU dose of heat-inactivated vaccine enhanced the antibody responses. These results demonstrate that i.d. administration of an inactivated VZV vaccine can be an efficient mode of immunization against VZV.

Varicella-zoster virus (VZV) causes varicella by primary infection and herpes zoster by reactivation of the latent virus in the sensory ganglia of infected individuals. After primary infection, the immune response comprises VZV-specific antibody and T cell-mediated immunity (CMI), which are important for recovery from varicella. T cell responses are necessary to control latent VZV in the sensory ganglia. A lack or a declining level of CMI to VZV has been associated with a higher risk of development of herpes zoster (1).

A varicella vaccine consisting of live, attenuated strain OKA (vOKA) has been developed in Japan and licensed for mass vaccination in Japan, South Korea, the United States, and several European countries or recommended for only selected groups of the population in other countries (2, 3). To prevent herpes zoster, a zoster vaccine containing 14 times as many PFU of vOKA than the varicella vaccine was developed and licensed for the vaccination of immunocompetent subjects older than 60 years in the United States in 2006 (4). Varicella and zoster vaccines are administered by the subcutaneous (s.c.) route. However, vaccination of immunocompromised individuals with live VZV vaccines can be problematic and different strategies for safe immunization need to be explored (5).

Several clinical studies have indicated that the use of a heat-inactivated VZV vaccine is an alternative mode of immunization of immunocompromised individuals. Triple vaccination of bone marrow transplant patients with a heat-inactivated varicella vaccine administered s.c. decreased the severity of herpes zoster (6) and four s.c. doses of a heat-inactivated zoster vaccine proved safe and immunogenic in patients with tumor malignancy, HIV-infected individuals, or hematopoietic stem cell transplant recipients (7). When healthy elderly subjects were immunized s.c. with a single dose of either live or heat-inactivated varicella vaccine, there were no differences in antibody responses or IFN-γ production by peripheral blood mononuclear cells (8). These data indicated that a heat-inactivated VZV vaccine might be useful in preventing herpes zoster. However, protection against herpes zoster following immunization with either a live or heat-inactivated vaccine is not optimal and a more potent antigenic stimulus is needed to improve the efficacy of the vaccine in high-risk patients (9).

The skin is a highly immunogenic organ (10). Noninvasive, needle-free liquid jet injection of liquid or powder into the skin has been used in clinical trials for immunization against viral infections (11–13). The barrier structure and thickness of the stratum corneum, the outermost layer of the skin, are similar in guinea pigs and humans (18.6 and 18.2 μm, respectively) (14), and thus, the i.d. route of immunization and the effectiveness of a potential i.d. delivery device for humans can be tested in guinea pigs. Moreover, the parental OKA strain is attenuated in human
and guinea pig fibroblast cells and vOKA is replicated in guinea pig cells (3). The i.d. injection of guinea pigs with VZV resulted in the infection of neurons in the dorsal root ganglia and gut, indicating viral transport and replication (15). Similarities or differences between the immune responses induced by a live or heat-inactivated VZV vaccine can therefore be tested in guinea pigs.

We investigated the VZV-specific immune responses of guinea pigs to the i.d. or s.c. administration of a live or heat-inactivated VZV vaccine. The expression of IFN-γ, granzyme B, and perforin mRNAs and IFN-γ protein production in the splenocytes of the immunized animals were measured. The antibody responses in the sera were compared by VZV glycoprotein-specific enzyme-linked immunosorbent assay (gpELISA) and virus neutralization assay.

MATERIALS AND METHODS

Study design. All protocols were approved by the Laboratory Animal Care Committee of the NCE. Guinea pigs were immunized with the 1/5 or full human dose of Varilrix VZV vaccine (i.e., 399 or 1,995 PFU, respectively) in live or heat-inactivated form administered i.d. or s.c. To ensure that equivalent amounts of vaccine were administered into the skin by the device, designed at the National Center for Epidemiology (NCE), in the first set of experiments, the VZV DNA contents of skin biopsy specimens obtained at different times after i.d. administration of the vaccine were determined by real-time quantitative PCR (qPCR). In the second series of experiments, guinea pigs were immunized with the 399- or 1,995-PFU dose of the vaccines, in live or heat-inactivated form, administered i.d. or s.c., and for CM1 responses, IFN-γ, granzyme B, and perforin mRNA expression in the VZV-stimulated splenocytes was determined by real-time quantitative reverse transcription-PCR assay (qRT-PCR). IFN-γ protein levels in the supernatants of the splenocytes were measured by ELISA, and the correlation between the expression of IFN-γ mRNA and the production of IFN-γ protein was investigated. VZV-specific antibody production was measured by gpELISA and virus neutralization assay. Nonimmunized animals were used as controls.

Vaccines. The VZV vaccine (Varilrix, GlaxoSmithKline, Rixensart, Belgium) contains 10^3.3 PFU/0.5 ml of live attenuated VZV (vOKA) propagated in MRC5 human diploid cells. Varilrix is for s.c. administration only. We prepared the heat-inactivated vaccine by treatment of the live vaccine at 56°C for 30 min. To confirm the loss of infectivity on heat inactivation, MRC5 cells (Medical Research Council, London, United Kingdom) were infected with the heat-inactivated or live vaccine and the expression of VZV antigens was tested by immunofluorescence assay with a mouse monoclonal antibody specific for VZV nucleopasid (LifeSpan BioSciences Inc., Seattle, WA) and VZV-positive human sera obtained from the Herpes Diagnostics Laboratory of the NCE and fluorescein isothiocyanate (FITC–anti-mouse (Trinity Biotech, Wicklow, Ireland) or –anti-human (DakoCytomation, Glostrup, Denmark) antibody conjugate, respectively. The cells infected with the heat-inactivated virus were completely negative for VZV antigens, while the cells infected with the live vaccine expressed these antigens abundantly.

Vaccine administration by the i.d. route. A needle-free liquid jet injection-based device that administers liquid into the skin under pressure was developed at the NCE (NCE device). Hartley guinea pigs 6 to 8 weeks old were used for the experiments (LAB-ÁLLBt, Budapest, Hungary).

qPCR detection of VZV DNA in guinea pig skin biopsy specimens. Thirteen animals received 0.1 ml of the Varilrix vaccine (399 PFU) by the i.d. route, and 11 animals received the heat-inactivated vaccine similarly. Skin punch biopsy samples (3 mm in diameter) from the site of vaccine administration were obtained from two or three animals/time point immediately after vaccination (day 0) and 2, 4, 7, and 14 days later and processed for DNA extraction and qPCR. Briefly, the skin biopsy specimens were pulverized with liquid nitrogen with a mortar and pestle and 600 μl of RLT-plus buffer was added to this powder. The homogenate was vortexed for 1 min and then centrifuged for 3 min at 12,000 × g. DNA was isolated from the supernatant according to the manufacturer’s instructions (Allprep DNA/RNA kit; Qiagen GmbH, Hilden, Germany). The qPCRs were carried out in a 10-μl final volume with a LightCycler 480 II thermostycler (Roche, Rotkreuz, Switzerland). The PCR mixture contained 2.5 μl of a DNA sample and 7.5 μl of a combination of 0.4 μl (100 μM) each of the forward and reverse primers, 0.2 μl (200 μM) of the probe, 5 μl of PCR master mix, and 1.5 μl of RNase-free distilled water. The PCR conditions were as follows: 95°C hot start for 15 min, followed by 50 amplification cycles of 95°C for 10 s, annealing at 50°C for 20 s, and 72°C for 10 s. The data were analyzed by the LightCycler 480 II system. DNA content was taken as the cycle threshold (Ct) value, i.e., the number of PCR cycles at which the amount of amplified target was detected above the threshold value. The VZV primers used amplified open reading frame 29 (early DNA binding protein) of the VZV genome with sense primer 5′-CGT ACA CGT ATT TTC AGT CCT CTT-3′, antisense primer 5′-GCG TTA GAC GTG TAG TTA ACA-3′, and probe 6-carboxyfluorescein (FAM)–GCC GTG GAG CCG GTG CTC GAA A–6-carboxytetramethylrhodamine (TAMRA) (16). The primers and probe for the reference housekeeping RNase P gene (Genbank accession no. XM_005000167.1) were designed at the NCE (sense primer 5′-GGA TTG AGA CAG AGC G-3′; antisense primer, 5′-GAG CGG CAG TTT CCA CCA TT-3′; probe, FAM-TTC TGA TCT GAA GGG TCG TGG TG-TAMRA). Primer efficiencies and the dynamic range of the target gene assay were determined in triplicate with serial dilutions of DNA isolated from biopsy samples obtained immediately after the vaccination of a guinea pig. ΔCT(Cv, VZV minus Cc, RNase P) was calculated for each DNA dilution, and the log DNA dilution was plotted versus ΔCT. The slope of the line was close to zero; hence, the efficiencies of the target and reference genes were similar. The 2−ΔΔCT calculation ΔAGCv = (CtTarget − CtRNase P)time s = (CTTarget − CRNase P)time zero was used for relative quantification (17). The mean value of the samples obtained immediately (time zero) after i.d. administration of the vaccine was used as a reference.

Immunization of guinea pigs. Four to six animals per group were immunized i.d. or s.c. with either the 399-PFU or the 1,995-PFU dose of the live or heat-inactivated vaccine. The animals were sacrificed 4 weeks after vaccine administration, and blood and splenocytes were obtained. Nonimmunized guinea pigs served as controls. Six guinea pigs vaccinated i.d. with the 399-PFU dose of heat-inactivated vaccine received an i.d. booster with the same vaccine preparation 23 weeks later. Blood and splenocytes were obtained 4 weeks after the booster administration.

VZV antigen preparation and stimulation of splenocytes. The VZV antigen for the stimulation of splenocytes was prepared as described earlier (18), with some modifications. Briefly, guinea pig embryonic fibroblast cells from 28-day-old embryos of Hartley guinea pigs, grown in tissue culture flasks, were infected with the live vaccine and incubated in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% heat-inactivated serum obtained from healthy guinea pigs. On day 5 after infection, cells were scraped into phosphate-buffered saline (PBS)–sucrose–glutamate buffer containing 10% heat-inactivated guinea pig serum. A few drops of this suspension was processed for immunofluorescence testing with VZV-positive human serum to ensure the presence of VZV antigens in the guinea pig cells, and the cells expressed VZV antigens. The remaining suspension was sonicated on ice with a VibraCell 72434 (Bioblock Scientific, Illkirch, France) ultrasonic disintegrator (3 × 15 s) and clarified by centrifugation at 3,000 rpm for 10 min at 4°C, and the supernatant was aliquoted and frozen at −80°C until use. For splenocyte stimulation, immunized and nonimmunized guinea pigs were sacrificed and splenocyte suspensions were prepared. Splenocytes (2 × 10^6 per 0.6 ml) were stimulated with a VZV antigen preparation at 10 μg/ml protein for 24 h.

Total RNA isolation and RT. Total RNA was extracted from stimulated splenocytes with the RNeasy Plus minikit and treated with the RNase-Free DNase set to remove genomic DNA contamination according to the manufacturer’s instructions (Qiagen GmbH, Hilden, Germany). The total RNA was eluted with 50 μl of RNase-free water, and the mRNA
was reverse transcribed with the Transcriptor First Strand cDNA synthesis kit in accordance with the manufacturer’s instructions (Roche, Mannheim, Germany). The resulting cDNA was stored at −80°C until qRT-PCR assay.

Detection of mRNA expression by qRT-PCR assay. IFN-γ, granzyme B, and perforin mRNAs were quantified by qRT-PCR assay. The qRT-PCRs were carried out as described for qPCR but with 2.5 μl of cDNA sample in the reaction mixture at an annealing temperature of 60°C. The primers and probes for IFN-γ were as described earlier (sense primer, 5'-CAT GAA CAC CAT CAA GGA ACG AAT-3'; antisense primer, 5'-TTT GAA GTA GTT TTT TAA ACG CC-3'; probe, FAM-CTC AAC AGC AGC AAC AAG GTG C-TAMRA) (19). The primers and probes for granzyme B (GenBank accession no. XM_003460665.1) and perforin (GenBank accession no. XM_003473749.1) target genes were designed at the NCE (granzyme B sense primer, 5'-TCC AGA GGG AAC ATA CCC AG-3'; antisense primer, 5'-GTA AGA GTT CCT CAC ACT TTC-3'; probe, FAM-CAC TGC AGG AAG TGG AGA TAG-TAMRA; perforin sense primer, 5'-CAG CAG AAG AGG CCC AAT GA-3'; antisense primer, 5'-TGG AAG ATG GTT ACC GTC AG-3'; probe, FAM-TGG CAC GGC CTC GCA GTC ATC ACC TAMRA). The RNase P primers and probe are described for qPCR. The product size was <150 bp for each amplicon. Each measurement was performed in triplicate. Primer efficiencies and the dynamic range of each target gene assay (IFN-γ) quantification was performed by the 2^-ΔΔCt method [20]. The slopes of the lines were close to zero. Relative quantification was performed by the 2^-ΔΔCt method (ΔΔCt = (CtTarget - CTRNase P)Immunized animal - (CtTarget - CTRNase P)Nonimmunized animal), i.e., the relative expression of the target gene was calculated as the ratio of the mean Ct value of the target gene (IFN-γ, granzyme B or perforin) to that of the housekeeping gene (RNase P) in each sample in relation to a reference sample (the mean of the samples obtained from nonimmunized animals).

ELISA to test the IFN-γ protein level and its correlation with the expression of IFN-γ mRNA. Splenocyte supernatants were assayed in duplicate with an ELISA kit (Guinea Interferon γ ELISA kit; BlueGene Biotech, Shanghai, China) according to the instructions of the manufacturer. Optical densities (ODs) at 450 nm of samples were read with an ELISA plate reader (PR 3100; Bio-Rad, Budapest, Hungary). The correlation between the expression of IFN-γ mRNA in stimulated splenocytes and the IFN-γ protein level in the supernatant of the splenocyte cultures was determined. A guinea pig was considered IFN-γ- or mRNA reactive if the mRNA expression level was ≥10-fold higher than that of nonimmunized animals. IFN-γ protein reactivity was considered to exist when the IFN-γ concentration (pg/ml) of the supernatant of splenocyte cultures from immunized animals was higher plus 3 interquartile ranges (IQRs) than that of nonimmunized animals.

VZV-specific antibodies in the sera of immunized animals as determined by VZV gpELISA and virus neutralization assay. VZV glycoprotein-specific IgG antibodies were determined in duplicate in serial dilutions of the sera of four to six guinea pigs per group with ELISA microplates coated with highly purified VZV glycoproteins (EUROIMMUN anti-VZV glycoprotein ELISA [IgM] kit; EUROIMMUN AG, Lübeck, Germany), horse-radish peroxidase–anti-guinea pig IgG conjugate (DakoCytomation, Glostrup, Denmark), and tetramethylbenzidine–H2O2 substrate from the EUROIMMUN ELISA kit. ODs at 450 and 620 nm were measured. The serum titers were determined as the reciprocal of the highest dilution at which the OD value exceeded 0.2 after subtraction of the no-serum blank value. The mean negative test OD plus 3 standard deviations was used to establish a cutoff value by repeated testing of sera from 10 nonimmunized animals. The negative-control sera produced an OD reading of <0.05 in each test.

For the determination of neutralization antibody titers, a plaque re-
The expression of perforin mRNA was significantly higher only in animals that received the heat-inactivated vaccine i.d. than that in nonimmunized animals ($P < 0.0051$) (Fig. 3C).

IFN-γ protein production of guinea pig splenocytes after a single i.d. or s.c. administration of a 1,995-PFU dose of the heat-inactivated or live vaccine s.c. mRNA expression does not necessarily indicate protein expression. We therefore used an ELISA to test the IFN-γ protein production of splenocytes from animals immunized with the heat-inactivated form of the vaccine i.d. or s.c. and compared the results with those of splenocytes obtained from guinea pigs immunized with the live vaccine s.c., the currently accepted form and route of VZV vaccination (Table 1). The content of IFN-γ protein ($P < 0.05$) and the fold increase in the expression of IFN-γ mRNA were significantly higher in animals immunized by the i.d. route ($P < 0.005$) or by the s.c. route ($P < 0.05$) than in nonimmunized animals. Whereas the fold increase in IFN-γ mRNA in animals that received the heat-inactivated vaccine by the i.d. route was significantly higher than that in animals that received this vaccine s.c. ($P = 0.025$) (see also Fig. 3A), the difference between the IFN-γ protein contents in the supernatants was not significant. Nevertheless, the animals classified as mRNA reactive mirrored the animals classified as IFN-γ protein reactive and the correlation coefficient of the median fold increase in mRNA expression and IFN-γ protein production was 0.88.

VZV glycoprotein-specific and virus-neutralizing antibody levels after VZV vaccination by the i.d. or s.c. route with the live or heat-inactivated form. The antibody production in all groups of guinea pigs immunized with the 399- or 1,995-PFU dose and in the groups immunized with the 399-PFU dose of heat-inactivated vaccine given twice i.d. was tested by a gpELISA (Table 2). The 1,995-PFU dose of vaccine proved to induce similar VZV glycoprotein-specific IgG antibody titers in guinea pigs immunized with the live or heat-inactivated vaccine either i.d. or s.c., with the nonsignificantly highest level in the animals immunized with the live vaccine s.c. The heat-inactivated vaccine induced a stronger antibody response when administered i.d. than when given s.c.,
but the difference was not significant. The 399-PFU dose of heat-inactivated vaccine administered i.d. induced a significantly lower titer of glycoprotein-specific IgG antibodies than the 1,995-PFU dose given similarly once ($P < 0.05$). The i.d. booster inoculation with the 399-PFU dose of heat-inactivated vaccine produced an antibody response significantly greater than that of the animals immunized with one dose of the same vaccine ($P < 0.005$). The neutralization titers showed no intergroup differences in animals immunized with the 1,995- or 399-PFU dose of the vaccines. No neutralization activity was observed in the animals receiving the 399-PFU dose of heat-inactivated vaccine i.d. once, while the animals receiving the same vaccine i.d. two times developed a neutralization titer of 1:32.

**DISCUSSION**

In humans, the i.d. route is routinely used for *Mycobacterium bovis* BCG and recommended for rabies vaccination in some countries, and influenza vaccines for i.d. administration are commercially available. Clinical trials with various other viral vaccines administered i.d. yielded promising data (11, 12, 21). However, poor immune responses have also been reported after i.d. rather than intramuscular administration of the hepatitis B surface antigen vaccine (22). We are not aware of investigations of immune responses to VZV vaccines delivered i.d.

A VZV-specific skin test representing delayed-type hypersensitivity was introduced earlier for testing of CMI in humans with past VZV infection or in humans and guinea pigs after VZV immunization, and the results were compared with those of other assays of immune responses (23–25). The accumulation of CD4+ T cells and CD4+ regulatory T cells at the site of VZV antigen challenge in the skin has recently been described (26). VZV-specific CMI assays have also determined the IFN-$\gamma$ protein production of immune cells, i.e., the VZV responder cell frequency by enzyme-linked immunospot assays, flow cytometry (7, 27), or

**FIG 3** IFN-$\gamma$, granzyme B, and perforin mRNA expression in stimulated splenocytes from guinea pigs immunized with the 1,995-PFU dose of VZV vaccine given in different forms and by different routes. IFN-$\gamma$ (A), granzyme B (B), and perforin (C) mRNA expression in splenocytes from immunized animals relative to that in splenocytes from nonimmunized animals is shown. HI, heat inactivated; L, live; *, $P < 0.05$; **, $P < 0.005$.

**TABLE 1** Correlation between IFN-$\gamma$ mRNA expression and IFN-$\gamma$ protein production by stimulated splenocytes from guinea pigs vaccinated with the 1,995-PFU dose of heat-inactivated vaccine administered i.d. or s.c. or with the live vaccine given s.c.

| Vaccine and route | IFN-$\gamma$ protein pg/ml median (range, IQR) | No. of IFN-$\gamma$ protein-reactive/total no. of animals | IFN-$\gamma$ mRNA fold increase median (range, IQR) | No. of IFN-$\gamma$ mRNA-reactive/total no. of animals |
|------------------|---------------------------------|-------------------------------------------------|---------------------------------|-------------------------------------------------|
| HI i.d. | 291 (264–328, 18) | 5/5 | 326 (77–1,461, 137) | 6/6 |
| HI s.c. | 273 (209–300, 73) | 5/5 | 119 (71–223, 109) | 5/5 |
| L s.c. | 353 (273–401, 42) | 4/4 | 461 (142–1,941, 674) | 4/4 |
| N | 154 (145–163, 9) | NA | 1 | NA |

a Criteria for animals reactive for IFN-$\gamma$ mRNA or for IFN-$\gamma$ protein are described in Materials and Methods.
b HI, heat inactivated; L, live; N, nonimmunized animals.
c $P < 0.05$ compared with nonimmunized animals.
d $P < 0.005$ compared with nonimmunized animals.
e $P < 0.05$ compared with i.d. administration.
f NA, not applicable.
cells in VZV infections are controversial. NK cells and possibly toxic T lymphocyte (CTL) or granzyme B production by immune responses, but VZV-specific CD8\(^+\) T cell responses were not detected in VZV-primed mice (35).

No data appear to have been reported concerning the production of perforin in the course of varicella, herpes zoster, or immunization with a VZV vaccine. However, perforin mRNAs were detected by RT-PCR in human CD4\(^+\) CTLs specific for herpes simplex virus (36).

Our results revealed an increased expression of granzyme B mRNA in immunized guinea pigs, as suggested by earlier studies with humans (32, 33). In addition, we demonstrated for the first time an increased expression of perforin mRNA after immunization with live or heat-inactivated vaccine, indicating the activation of VZV-specific CTLs. Interestingly, the expression of IFN-γ, granzyme B, and perforin mRNAs was not significantly higher in splenocytes obtained from live-virus-immunized animals than in those immunized with heat-inactivated vaccine by the same route. VZV peptides from the heat-inactivated vaccine were probably phagocytosed by antigen-presenting cells in the guinea pig skin and cross-presented to T lymphocytes in the lymph nodes, resulting in the activation of lymphocytes to express IFN-γ, granzyme B, and perforin mRNAs.

Since outbred Hartley guinea pigs were used in the immunization studies, the CMI responses of some of the animals may have been weak because of the inability of certain major histocompatibility complex (MHC) types of antigen-presenting cells to recognize the VZV stimulus composition and to activate CD8\(^+\) lymphocytes. In some cases, no significant difference was observed because of the broad range of cellular immune responses in a given group of animals, perhaps owing to their outbred nature. MHC restriction of T cell responses to VZV in guinea pigs was reported earlier (37). Nevertheless, VZV vaccination is destined for the outbred human population and similar experiments with inbred animal strains would therefore not be helpful.

VZV-specific antibodies have negligible roles in protection against reactivation of the latent virus in the ganglia and the development of zoster. However, VZV-specific antibody production seems important in protection against infection. Maternal antibodies and the passive transfer of VZV-specific antibodies, for instance, can prevent or mitigate the development of varicella after exposure to VZV (38, 39). Thus, the production of a high antibody level by VZV vaccination is desirable in either healthy or immunocompromised individuals. The VZV gpELISA method seems to perform comparably to the virus neutralization assay (40), and the presence of neutralizing antibodies is associated with a positive fluorescent antibody to membrane antigen test result, which has been shown to correlate with protection against clinical varicella (41). We have demonstrated that VZV glycoprotein-specific antibodies are produced in guinea pigs following i.d. or s.c. administration of a 399- or 1,995-PFU dose of heat-inactivated vaccine, the 1,995-PFU dose being significantly more effective (P < 0.005). The antibody level was significantly increased after an i.d. booster inoculation with the 399-PFU dose of heat-inactivated vaccine (P < 0.005). The results showed a concordance between the titers in the gpELISA and the neutralization assay, as was observed previously (40). The mechanism by which the i.d. administration of the heat-inactivated vaccine at a dose of 1,995 PFU induces a significantly higher level of CMI but nonsignificantly higher titers of antibodies than the same vaccine given s.c. is not clear.

### TABLE 2 VZV gpELISA and virus neutralizing-antibody titers in the sera of immunized guinea pigs

| Dose (PFU), vaccine, and route | Endpoint titer of antibody to VZV glycoproteins | Neutralizing-antibody titer |
|------------------------------|--------------------------------------------------|-----------------------------|
| 399                          |                                                  |                             |
| HI i.d.                      | 200                                              | <4                          |
| HI s.c.                      | 168                                              | <4                          |
| L.i.d.                       | 159                                              | <4                          |
| L.s.c.                       | 425                                              | 4                           |
| HI i.d. + HI i.d.            | 21,544\(^b\)                                     | 32                          |
| 1,995                       |                                                  |                             |
| HI i.d.                      | 131,950\(^a\)                                    | 128                         |
| HI s.c.                      | 83,255                                           | 64                          |
| L.i.d.                       | 100,000                                          | 80                          |
| L.s.c.                       | 263,901                                          | 138                         |

\(^a\) VZV glycoprotein-specific IgG antibody titers are expressed as the reciprocal of the highest dilution of serum in which the OD exceeded 0.2. Neutralization titers are expressed as the reciprocal of the highest dilution that reduced the number of plaques by 50%. Geometric mean values are shown.

\(^b\) P < 0.005 compared with HI i.d.

\(^c\) P < 0.05 compared with HI i.d.

\(^d\) HI i.d. + HI i.d.

\(^e\) P < 0.005 compared with HI i.d.

IFN-γ ELISA of supernatants of ex vivo-stimulated lymphocytes (28). The qRT-PCR assay allows highly sensitive testing of the mRNA expression of selected genes of importance in humans (29) and animals (30). Measurements of the expression of the mRNAs for IFN-γ, granzyme B, and perforin, which are encoded by the important cellular genes controlling CMI, do not appear to have been utilized in evaluations of CMI responses to VZV vaccine candidates administered in various forms and by various routes.

In our study, qRT-PCR proved to be a highly sensitive method for the determination of IFN-γ mRNA expression. Importantly, the i.d. delivery of a 1,995-PFU dose of heat-inactivated VZV vaccine induced a significantly higher level of IFN-γ mRNA in stimulated splenocytes than the same vaccine given s.c. Splenocytes from all animals expressing IFN-γ mRNA produced IFN-γ protein with a correlation coefficient similar to that obtained in subjects with active tuberculosis (31) or animals infected with M. bovis (30).

The available data concerning the role of VZV-specific cytotoxic T lymphocyte (CTL) or granzyme B production by immune cells in VZV infections are controversial. NK cells and possibly primed CD8\(^+\) cells produced higher levels of granzymes A and B in some children with severe varicella disease than in children with a mild or moderate form of the disease (32). CTL responses were detected in healthy adults immunized with live attenuated varicella after exposure to VZV (38, 39). Thus, the production of a high antibody level by VZV vaccination is desirable in either healthy or immunocompromised individuals. The VZV gpELISA method seems to perform comparably to the virus neutralization assay (40), and the presence of neutralizing antibodies is associated with a positive fluorescent antibody to membrane antigen test result, which has been shown to correlate with protection against clinical varicella (41). We have demonstrated that VZV glycoprotein-specific antibodies are produced in guinea pigs following i.d. or s.c. administration of a 399- or 1,995-PFU dose of heat-inactivated vaccine, the 1,995-PFU dose being significantly more effective (P < 0.005). The antibody level was significantly increased after an i.d. booster inoculation with the 399-PFU dose of heat-inactivated vaccine (P < 0.005). The results showed a concordance between the titers in the gpELISA and the neutralization assay, as was observed previously (40). The mechanism by which the i.d. administration of the heat-inactivated vaccine at a dose of 1,995 PFU induces a significantly higher level of CMI but nonsignificantly higher titers of antibodies than the same vaccine given s.c. is not clear.
A heat-inactivated vaccine administered i.d. might be efficient and safe in immunocompromised children and adults. This mode of VZV vaccination may also be relevant in the case of elderly immunocompetent individuals, who currently receive an expensive vaccine that contains a large dose of live virus. The boost of immunity in the elderly to prevent zoster by the i.d. route with a heat-inactivated vaccine might be preferable because it uses less virus and gives stronger cellular responses.

Our study has limitations. (i) The expression of IFN-γ, granzyme B, and perforin mRNAs may follow different kinetics after immunization or after the VZV-specific stimulation of splenocytes, and the expression level may even differ in different tissues of the immune system. Marked diversity has been reported in the perforin, granzyme B, and IFN-γ mRNA expression profiles of activated CD8 T cells from influenza virus-infected mice, with different frequencies in CD8+ populations obtained from the lungs, lymph nodes, and spleen (42). We determined mRNA expression at one time point after immunization and harvested splenocytes at one time point after stimulation to determine IFN-γ, granzyme B, and perforin mRNA expression. Thus, it is possible that the conditions in our experiments were not optimal for each mRNA tested. Nevertheless, we could determine the expression of the mRNAs of these immune effector molecules in guinea pigs immunized with VZV vaccines, including the heat-inactivated vaccine given i.d. (ii) We did not test the kinetics of IFN-γ mRNA and protein expression after the in vitro stimulation of splenocytes with a VZV antigen preparation. A time difference in IFN-γ mRNA expression and IFN-γ protein synthesis has been reported after in vitro stimulation of human blood samples with Mycobacterium tuberculosis antigen (31). Further studies are needed to determine the kinetics, levels, and correlations of the expression of mRNAs and proteins of cellular genes involved in the CMI of VZV-immunized individuals. However, the physiological significance of small mRNA or protein level changes is not clear. (iii) We did not carry out intracellular cytokine staining assays to immunophenotype the IFN-γ, granzyme B, and perforin responder cells. CD4+ proliferative responses are believed to correlate with protection against zoster (43), but CD8+, CD8-+, and NK cells may be involved differentially in the production of IFN-γ, granzyme B, and perforin in individuals immunized i.d. or s.c. with the live or heat-inactivated vaccine.

In conclusion, our data show that (i) the guinea pig is a suitable species in which to test the i.d. route of immunization; (ii) IFN-γ, granzyme B, and perforin mRNA expression is a highly sensitive measure of VZV-specific CMI; (iii) a heat-inactivated VZV vaccine administered i.d. can induce a higher level of cellular anti-VZV responses in guinea pigs than the same vaccine given s.c.; and (iv) the VZV glycoprotein-specific IgG antibody responses and the virus-neutralizing activity of the sera are similar in animals immunized i.d. or s.c. with live or heat-inactivated vaccine. The findings following the use of a heat-inactivated VZV vaccine administered i.d. warrant further investigation. The optimal dose of the vaccine should be determined. Information on the duration of the immune response to a single or booster i.d. or s.c. heat-inactivated VZV vaccine administration would be important. Moreover, a favorable effect of adjuvants is to be expected and should be explored.

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Heat-Inactivated VZV Vaccine Given Intradermally
Correction for Sarkadi et al., High-Level Cellular and Humoral Immune Responses in Guinea Pigs Immunized Intradermally with a Heat-Inactivated Varicella-Zoster Virus Vaccine

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