Variella-zoster virus (VZV) causes chickenpox and becomes latent in sensory ganglia. Herpes zoster is caused by the reactivation of latent VZV in sensory trigeminal and dorsal root ganglia. Reactivated VZV replicates in the skin and produces the characteristic herpes zoster rash accompanied by acute pain [1]. The incidence and severity of herpes zoster increase with age because of age-related waning of VZV-specific cell-mediated immunity (CMI); more than half of persons who develop herpes zoster are >60 years old [2]. Live attenuated VZV vaccine can boost a VZV-specific CMI that has decreased as part of the aging process, thereby preventing the reactivation of VZV and reducing the severity of herpes zoster [3–7]. Furthermore, vaccination with inactivated varicella is effective for reducing the risk of herpes zoster in immunocompromised patients [8]. Therefore, screening individuals who are susceptible to herpes zoster by monitoring the state of their immunity to VZV and boosting their immunity by vaccination could be an effective way of decreasing herpes zoster morbidity.

The varicella skin test has long been used to assess CMI to VZV [9, 10]. With this test, an individual’s susceptibility to VZV can be easily judged by an erythematous change 24–48 h after intradermal antigen injection. It is a simple and safe way to assess CMI to VZV, because no special skill or laboratory equipment is needed. Recently, an interferon (IFN)–γ enzyme-linked immunospot (ELISPOT) assay was reported as a novel method for assessing CMI to VZV [11, 12]. Because this assay directly measures the number of T cells secreting IFN-γ after stimulation with VZV antigen, it is extremely sensitive and specific compared with other assays, such as the lymphoproliferative activity assay [13,
Although both the varicella skin test and IFN-γ ELISPOT assay can be used to measure CMI to VZV, a correlation between the results of these two tests has not been clearly shown. In this study, we performed both assays to measure CMI to VZV in 151 healthy volunteers and evaluated the validity of these methods.

**METHODS**

**Study design.** In the same session, a VZV skin test was performed and a blood specimen collected for the IFN-γ ELISPOT assay and ELISA. Serum samples were collected separately for each participant in the study. This study was approved by the ethical committees of all the involved institutions. Written informed consent was obtained from all the enrolled volunteers.

**Population.** In 151 healthy volunteers, we studied CMI to VZV using both the IFN-γ ELISPOT assay and the VZV skin test and humoral immunity using glycoprotein antigen–based ELISA (gpELISA). The characteristics of the study population are shown in tables 1 and 2. The mean age was 49.1 years (range, 23–66 years), and 83 subjects were male (55%). Of the 151 subjects, 12 had a history of herpes zoster, 10 had received varicella vaccination, and 5 had both. Sixteen subjects were aged 20–29 years (10.6%), 26 were aged 30–39 years (17.2%), 18 were aged 40–49 years (11.9%), 73 were aged 50–59 years (48.3%), and 18 were aged 60–69 years (11.9%).

**Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood.** Whole blood was collected from donors into Venoject heparin-containing tubes (Terumo). The blood was diluted with PBS without calcium and magnesium and layered on top of a Ficoll solution. The tubes were spun at 880 g for 25 min at 20°C, and the buffy layer containing the PBMCs was collected. The cells were washed twice with PBS, resuspended in stock solution consisting of 90% heat-inactivated fetal bovine serum (Gibco BRL) and 10% dimethyl sulfoxide (Wako), and placed into a Bicell cryogenic freezing container (Nihon Freezer). The freezing container was stored at −80°C overnight, and the frozen cell samples were then transferred to liquid nitrogen (vapor phase) for long-term storage.

**Cell sample preparation from frozen PBMCs.** Complete medium, consisting of RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL), 1 mmol/L l-glutamine, 20 µg/mL gentamicin, and 50 µmol/L β-mercaptoethanol, was warmed to room temperature and supplemented with Benzonase (Novagen) to a final concentration of 40 U/mL. The frozen PBMCs were thawed at 37°C and suspended in the Benzonase-supplemented complete medium. The cells were washed again, resuspended in complete medium without Benzonase, and counted. After another wash, cells were resuspended in complete medium without Benzonase at a concentration of 4 × 10^6 cells/mL and used for assays.

**IFN-γ ELISPOT assay.** The wells of a 96-well MultiScreen HA plate (Millipore) were coated with 100 µL of an anti–human Table 1. Characteristics of 151 healthy volunteers.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), years</td>
<td>49.1 (23–66)</td>
</tr>
<tr>
<td>Male sex, no.</td>
<td>83</td>
</tr>
<tr>
<td>Case history, no. (%)</td>
<td>83</td>
</tr>
<tr>
<td>Herpes zoster</td>
<td>12 (7.9)</td>
</tr>
<tr>
<td>Varicella vaccination</td>
<td>10 (6.6)</td>
</tr>
<tr>
<td>Herpes zoster and vaccination</td>
<td>5 (3.3)</td>
</tr>
<tr>
<td>No history of zoster and/or</td>
<td>124 (82.1)</td>
</tr>
</tbody>
</table>

**NOTE.** Case history data were self-reported on a questionnaire administered at enrollment.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of subjects</th>
<th>Skin test positive, %</th>
<th>Skin test result, mm</th>
<th>ELISPOT count</th>
<th>gpELISA positive, %</th>
<th>gpELISA antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td>151</td>
<td>78.8</td>
<td>16.0</td>
<td>87.7</td>
<td>100</td>
<td>7891</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20–29 years</td>
<td>16</td>
<td>87.5</td>
<td>18.4</td>
<td>120.0</td>
<td>100</td>
<td>11,835</td>
</tr>
<tr>
<td>30–39 years</td>
<td>26</td>
<td>80.7</td>
<td>17.4</td>
<td>104.6</td>
<td>100</td>
<td>5763</td>
</tr>
<tr>
<td>40–49 years</td>
<td>18</td>
<td>83.3</td>
<td>17.6</td>
<td>106.9</td>
<td>100</td>
<td>9833</td>
</tr>
<tr>
<td>50–59 years</td>
<td>73</td>
<td>78.1</td>
<td>15.0</td>
<td>72.1</td>
<td>100</td>
<td>6906</td>
</tr>
<tr>
<td>60–69 years</td>
<td>18</td>
<td>66.7</td>
<td>14.2</td>
<td>79.0</td>
<td>100</td>
<td>9507</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>83</td>
<td>81.9</td>
<td>16.7</td>
<td>85.6</td>
<td>100</td>
<td>8680</td>
</tr>
<tr>
<td>Female</td>
<td>68</td>
<td>76.5</td>
<td>15.3</td>
<td>90.4</td>
<td>100</td>
<td>7101</td>
</tr>
</tbody>
</table>

**NOTE.** Subjects are stratified according to age or sex. For each stratum, average values are shown for the skin test, IFN-γ ELISPOT assay, and glycoprotein antigen–based ELISA (gpELISA). In the IFN-γ ELISPOT assay, 4 × 10^5 peripheral blood mononuclear cells were used. Positive skin test results were defined as reactions >10 mm (longest diameter), and positive gpELISA results were defined as antibody titers >50.
recombinant IFN-γ monoclonal antibody (clone 2G1; Endo-
gen) at a concentration of 3 μg/mL, overnight at 4°C. The wells were then washed 3 times with sterile PBS and blocked by adding 200 μL of complete medium followed by incubation at 37°C with 5% CO₂ for 1.5–2 h. The wells were then washed once with 100 μL of complete medium. Next, 100 μL of PBMC suspension at 4 × 10⁶ cells/mL was added to each well, followed by an equal volume of complete medium containing the VZV antigen. UV-
treated (5000 J/m²) varicella vaccine (Biken) containing 3 × 10⁴ pfu/mL was used as the VZV antigen. Phytohemagglutinin-L₄ (Wako) at 2 μg/mL was included as a positive control.

The assay plates were incubated for 40 h at 37°C in 5% CO₂ and 95% humidity. The plates were then washed 4 times with PBS containing 0.1% Tween 20 (ELISPOT wash buffer). A biotinylated anti–human recombinant IFN-
 PBS containing 0.1% Tween 20 (ELISPOT wash buffer). Next, 100 μL of streptavidin-horseradish peroxidase (BD), diluted 1:1000 in ELISPOT wash buffer, was added to each well, and the plates were incubated at room temperature for 45 min. The plates were washed 4 times with ELI-
SPOT wash buffer. Next, 100 μL of streptavidin-horseradish peroxidase (BD), diluted 1:1000 in ELISPOT wash buffer, was added to each well, and the plates were incubated at room temperature for 45 min. The plates were washed 4 times with ELI-
SPOT wash buffer, 100 μL of 3,3′,5,5′-tetramethylbenzidine–H₄ substrate (Moss) was added, and the spots were developed for 3 min at room temperature. The substrate was then removed from the wells, and the wells were rinsed with water to stop the reaction. The plates were allowed to air dry, and the spots were then enumerated using the KS ELISPOT system (Carl Zeiss) for au-
tomated plate scanning, imaging, and spot counting.

Results of gpELISA. VZV glycoprotein (VZVgp) was puri-
fied following the method of Provost et al. [16], using the VZV Oka vaccine strain and MRC-5 cells. A similarly prepared glyco-
protein extract of uninfected MRC-5 cells (MRC-5gp) was used as the negative control. The wells of 96-well ELISA plates were coated with purified VZVgp or MRC-5gp for 24 h at 4°C. The plates were washed 4 times with PBS supplemented with 0.05% Tween 20 (PBS-T) and then blocked for 24 h with PBS supple-
mented with 0.05% human serum albumin. After the plates were washed 4 times with PBS-T, serum standards, negative and posi-
tive control serum samples, and test serum samples, all diluted in PBS-T supplemented with 0.25% human serum albumin (di-
lution buffer), were added to the VZVgp- or MRC-5gp–coated wells. The first plates included a set of blank wells containing dilution buffer alone, to assess the background reactivity. The plates were incubated for 60 min at 37°C, followed by 4 washes with PBS-T. Human IgGs bound to the plates were detected with peroxidase-conjugated anti–human IgG for 60 min at 37°C, and the color was developed with 2,2′-azino-
obis(3-ethylbenzothiazoline-6-sulfonic acid). After the reaction was stopped, the optical density was read at 405 nm. The values were calculated by subtracting the optical density of MRC-5gp from that of VZVgp, and the values of test serum samples were referenced against a fitted standard curve deter-
mined from six 2-fold serial dilutions of the standard.

Figure 1. Determination of optimum conditions for the enzyme-linked immunoassay (ELISPOT) assay. A, Peripheral blood mononuclear cells (PBMCs) collected from 4 healthy volunteers (samples A, B, C, and D) were subjected to the ELISPOT assay. In each sample, 1 × 10⁶ to 6 × 10⁶ cells were stimulated with live of varicella-zoster virus (VZV) antigen (3 × 10⁴ pfu/mL), and the spots were automatically enumerated by the KS ELISPOT system (Carl Zeiss). B, Spot count of sample A stimulated with untreated live VZV antigen, heat-inactivated VZV antigen, or UV-inactivated VZV antigen. There was a significant reduction in spot-forming activity using heat-inactivated VZV compared with un-
treated VZV (P = .02, Student’s t test).

VZV skin test. One hundred microliters of VZV skin test antigen (Biken) was injected intradermally, and the erythema was measured 24 and 48 h later. The longest diameter is given in the figures and tables, because there was usually no significant difference between the longest and shortest diameters. The cri-
tera for scoring the reaction were defined as follows: 1 (negative), <10 mm; 2 (positive), ≥10 mm; and 3 (strongly positive), ≥10 mm with induration at 48 h.

Statistics. The effects of inactivation treatments were com-
pared using Student’s t test. Spearman’s correlation coefficient by rank test was used to analyze the correlation between skin test and ELISPOT assay results or between gpELISA and skin test
results. For the comparisons of the ELISPOT counts or gpELISA antibody titers with skin test scores, the Mann-Whitney U test was applied. Age-associated decline in skin test reactivity or ELISPOT count was assessed by the Wilcoxon signed rank test or Student’s t test.

**RESULTS**

**Determination of optimum conditions for the ELISPOT assay.** For the human IFN-γ ELISPOT assay, we first determined the optimum cell number. PBMCs were collected from 4 healthy volunteers and subjected to the ELISPOT assay. Each sample was plated at $1 \times 10^5$, $2 \times 10^5$, $4 \times 10^5$, and $6 \times 10^5$ cells/well in triplicate and stimulated with live VZV antigen solution or control solution. The spot count was performed automatically using the KS ELISPOT system. Mean (±SDs) spot counts were calculated after subtracting the control count from that of the VZV-stimulated sample wells. As shown in figure 1A, although the spot count increased almost in proportion to the cell number up to $4 \times 10^5$ cells/well, there was an obvious reduction at $6 \times 10^5$ cells/well, indicating that too high a cell number causes signal overlapping and hence a reduction in counting accuracy. On the other hand, at $1 \times 10^5$ cells/well, the spot count decreased to 0 or almost 0 in 2 samples (samples B and D), indicating that too low a cell number can result in an underestimation of the differences between samples. Therefore, we used $4 \times 10^5$ cells/well in the subsequent ELISPOT assays.

For antigen stimulation, among the concentrations tested ($0.3 \times 10^4$, $1 \times 10^4$, and $3 \times 10^4$ pfu/mL), $3 \times 10^4$ pfu/mL live varicella vaccine was determined to be effective.

Live VZV antigen stimulates CD4+ T cells to secrete IFN-γ, but it might also infect and replicate in T cells, which could also affect IFN-γ secretion. Therefore, to assess the amount of IFN-γ secretion caused by antigen stimulation, we examined the effects of inactivated VZV on the spot count (figure 1B). As shown in figure 1B, there was a significant reduction in spot-forming activity using heated VZV compared with untreated (live) VZV ($P = .02$, Student’s t test). On the other hand, up to 5000 J/m² UV-treated VZV had stimulation activity similar to that of the
control. In addition, we confirmed that UV treatment at 5000 J/m² completely inactivated the VZV (data not shown). Therefore, the subsequent ELISPOT assays were performed using UV-treated (5000 J/m²) VZV for antigen stimulation.

**Skin test reactivity, ELISPOT assay, and gpELISA.** Of the 151 subjects, 31 had a negative response by the skin test, but all of them had positive serologic results (antibody titer >50) by the gpELISA. The 31 subjects with negative skin test results included 4 vaccinees. As shown in table 2, both the rate of skin test positivity and the erythema diameter showed the tendency of an age-related decline, and the ELISPOT count showed the same pattern as the skin test. As expected, there were significant differences in the rate of skin test positivity and ELISPOT count between subjects in their 20s, 30s, or 40s and those in their 50s or 60s (figure 2A and 2B). The specific antibody titer obtained by the gpELISA remained relatively constant with increasing age (table 2 and figure 2C). There were no significant differences in the values when the results were stratified according to sex (table 2). Of the 151 subjects, 12 had a history of herpes zoster, 10 had received varicella vaccination, and 5 had both. We therefore investigated the correlation between these subjects and the remaining 124 individuals with respect to the ELISPOT and skin test results, but no relationship was seen.

**Correlation between skin test results and ELISPOT count.** All of the values obtained from the VZV skin test, ELISPOT assay, and gpELISA are presented as scatter plots in figure 3. The graphs show the correlation between the VZV skin test and the ELISPOT assay (figure 3A), the gpELISA and the ELISPOT assay (figure 3B), and the gpELISA and the VZV skin test (figure 3C). As shown in figure 3A, the approximation curve indicates a positive linear relationship between the VZV skin test result and the ELISPOT count. In contrast, the slope of the approximation curve is smaller in figure 3B and 3C, indicating only a weak correlation between the gpELISA and the ELISPOT assay or the VZV skin test.

Because the VZV skin test involves a rather complex in vivo reaction, minor variations in diameter may occur, depending on the physical condition of the subject. Therefore, the VZV skin test values were scored as follows: 1 (negative), <10 mm; 2 (positive), ≥10 mm; and 3 (strongly positive), >10 mm with induration. The average ELISPOT counts and gpELISA antibody titers were calculated for all of the subjects (figure 4). The ELISPOT counts increased in proportion with the skin test score (47.8 at score 1, 72.2 at score 2, and 105.3 at score 3), with P values of .02 and .03 for the comparison between scores 2 and 3 and between scores 1 and 2, respectively (Mann-Whitney U test), indicating a significant correlation between the skin test and ELISPOT results (figure 4A). Furthermore, Spearman’s correlation coefficient by rank test yielded a correlation coefficient of 0.43, indicating a moderately positive correlation between the 2 variables. On the other hand, the gpELISA results showed no significant differences when compared with the skin test scores (figure 4B) (correlation coefficient, 0.08). In addition, the coefficient was 0.21 when the gpELISA antibody titer was compared with the ELISPOT count, suggesting that the gpELISA antibody titer had only a weak or no linear association with the ELISPOT count or the skin test. Thus, the skin test results correlated well with the ELISPOT count but not with the gpELISA results.
DISCUSSION

Previous studies have indicated that a decline in CMI to VZV is closely correlated with the development of herpes zoster, although the mechanism of VZV reactivation in the neuron is not fully understood [7]. The waning of VZV-specific CMI occurs not only in seriously immunocompromised patients (such as those undergoing treatment for cancer or AIDS and recipients of organ transplants) but also in healthy elderly persons. A significant proportion of older subjects with herpes zoster develop postherpetic neuralgia, a chronic pain syndrome [17]. The pain is often long lasting and difficult to control; therefore, it has a severe impact on patients’ quality of life. Because the elderly population is rapidly increasing, there is an urgent need for effective ways to reduce the burden of this illness.

The zoster vaccine effectively reduces the risk and severity of herpes zoster, especially in the elderly [7]. However, it is still not clear how long the protection is maintained or whether repeated doses should be given—and, if so, at what intervals. Therefore, it has a severe impact on patients’ quality of life. Because the elderly population is rapidly increasing, there is an urgent need for effective ways to reduce the burden of this illness.

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A correlation between the skin test and the IFN-γ ELISPOT assay was indicated by a positive linear relationship in the scatter plot, although there were several outliers. The discrepancy between the skin test result and the ELISPOT count represented by the outliers might be due to the different assay methods: compared with the in vitro ELISPOT assay, the in vivo skin test reaction is much more complex and is more likely to be affected by the physical condition of the subject. It is also possible that, in some subjects, the skin test reaction was provoked nonspecifically by non-VZV components in the injected solution or that false-negative results might have been caused by an inadequate injection. Therefore, for higher accuracy, the skin test might require a control solution injection besides the VZV antigen injection to measure the nonspecific reaction.

In the ELISPOT assay, we observed that the size of the spots occasionally differed among the subjects examined, even among those showing the same number of spots. Although the spot number was used in the present analyses, the size of the spots also appeared to be an important marker for the strength of the im-
immune response. Therefore, further study is required to analyze the relationship between the spot size in this assay and the strength of the VZV-specific immune response.

The significant correlation between the results of the skin test and the ELISPOT assay was also indicated by the proportional change in the skin test score and the ELISPOT count (figure 4). Taken together, the results suggest that the skin test, like the ELISPOT assay, is valid for measuring CMI to VZV. Further study is needed to establish the immunoprotective threshold level of the skin test reaction for predicting an individual’s susceptibility to herpes zoster. Nevertheless, our findings indicate that the skin test, which is clearly the easiest method developed thus far for measuring CMI to VZV, can be broadly applied to screen people at high risk for herpes zoster and that the results may predict those at greatest risk for reactivation, providing informative criteria for varicella vaccination. Furthermore, the skin test should be useful in longitudinal studies to estimate the durability of the protection provided by vaccination.

Acknowledgments

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References