Prediction of Residual Immunity to Smallpox, by Means of an Intradermal Skin Test with Inactivated Vaccinia Virus

Sung-Han Kim, Ji-Whan Bang, Kyung-Hwa Park, Wan-Bum Park, Hong-Bin Kim, Nam-Joong Kim, Youngmee Jee, Haewol Cho, Myoung-don Oh, and Kang-Won Choe

1Department of Virology, National Institute of Health, and 2Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea

Background. Intradermal skin testing with inactivated vaccinia virus was evaluated for its prediction of residual immunity to smallpox.

Methods. An intradermal skin test was performed with heat-inactivated Lancy-Vaxinia. Two days later, the subjects were vaccinated with Lancy-Vaxinia. The skin lesions resulting from this vaccination were used as a surrogate marker of residual immunity to smallpox, and this surrogate marker was compared with the available indicators of susceptibility to smallpox.

Results. Of the 83 subjects, 30 (36%) showed the typical primary response after vaccination (i.e., absence of residual immunity), whereas 34 (41%) showed the typical revaccinee's response (i.e., presence of residual immunity); the remaining 19 (23%) had an indeterminate response and were excluded from the final analysis. The sensitivity and specificity of the intradermal skin test (induration size, \( \geq 4 \) mm) for prediction of residual immunity to smallpox were 85% and 97%, respectively, whereas those of a positive vaccinia-specific interferon-\( \gamma \)-producing T cell response (\( \geq 9 \) spot forming cells/10\(^6\) peripheral-blood mononuclear cells) were 32% and 63%, respectively, and those of a positive neutralizing antibody (titer, \( \geq 1:8 \)) were 79% and 80%, respectively.

Conclusion. The intradermal skin test appears to be a simple and reliable method for prediction of residual immunity to smallpox.

Residual immunity to smallpox after smallpox vaccination is an important issue for public health and vaccine development [1]. Recent in vitro studies have addressed this subject but have produced inconsistent results [1–4]. This inconsistency may be due to problems with the methods for evaluation of residual immunity, because assays for cell-mediated immunity are complex and difficult and because those for humoral immunity are laborious and not standardized. Therefore, a simple and reliable test of immune status with respect to smallpox is needed.

Because we cannot evaluate the present immunity of individuals by challenging them with actual variola virus, intradermal inoculation with vaccinia virus (i.e., smallpox vaccination) provides an alternative way to study human memory immune responses after poxvirus infection [5]; that is, smallpox vaccination “provides a unique human model of [poxvirus] infection to define the sequence of the immune responses that are essential for control of virus replication” [5, p. 1287]. Indeed, the skin lesions resulting from smallpox vaccination have long been considered the best indicators of “working” immunity to smallpox in an individual with a history of smallpox vaccination [6–9]; that is, the magnitude of immune memory is measured through the skin lesions after smallpox vaccination, in terms of the visible evidence of suppression of repli-
cation of vaccinia virus. We therefore used such skin lesions as a surrogate marker for residual immunity to smallpox.

The aim of the present study was to evaluate an intradermal skin test using inactivated vaccinia virus to predict residual immunity to smallpox. After administering such a skin test to subjects, we inoculated them with smallpox vaccine and examined their resulting skin lesions. We then compared the results of the intradermal skin test with the skin lesions resulting from the smallpox vaccination. We also compared the usefulness of this procedure with the other available indicators of residual immunity to smallpox—namely, the immediate vaccinia-specific interferon (IFN)–γ-producing T cell response and the titer of neutralizing antibody to vaccinia.

Subjects, Materials, and Methods

Study subjects. A randomized single-blind controlled trial comparing the efficacy of undiluted (1:1) and diluted (1:10) Lancy-Vaxina (Berna Biotech) was conducted at Seoul National University Hospital during February 2003–October 2004 [10]. Those of the subjects enrolled in that trial who agreed to have an intradermal skin test were eligible for the present study. The study was approved by the institutional review board of Seoul National University Hospital, and written, informed consent was obtained from each subject. Exclusion criteria for the study included the contraindications for smallpox vaccination that are noted in the US Centers for Disease Control and Prevention guidelines [11]. All subjects were asked to provide a history of vaccinia-virus vaccination, and vaccinia-virus vaccination scars were examined in all subjects.

Virus preparation and intradermal-skin-test protocol. Vaccinia virus was inactivated as described elsewhere [12, 13]. In brief, lyophilized Lancy-Vaxina was reconstituted with the diluent, in accordance with the directions on the package insert provided by the manufacturer. This reconstituted undiluted vaccine, without any serum or buffer, was inactivated by being heated for 1 h at 60°C. Each specimen of the heat-inactivated vaccine was inoculated, in duplicate, directly onto 12-well plates coated with BSC-40 cells. After adsorption for 1 h at 37°C, each monolayer was overlaid with methyl cellulose, in Eagle medium. The presence of any cytopathic effect was assessed every 24 h, for 2 days. After incubating for 2 days at 37°C, the plates were fixed with 10% formalin and were stained with 2% crystal violet to allow any resulting vaccinia plaques to be visualized; 2 independent experiments revealed no plaque formation in the specimens from the heat-inactivated vaccine solution. Finally, for the intradermal skin test, the solution was diluted, at a 1:20 ratio, with normal saline, at each use. This dilution ratio was selected after preliminary testing by us and also has been used in an earlier study [13]. We used the vaccinia-virus diluent as the control solution.

Skin testing was performed by use of the Mantoux technique, with insulin syringes, by intradermally inoculating 0.1 mL of the inactivated-vaccinia-virus solution into one forearm and the control solution into the other forearm. At 48 h after inoculation, a single investigator (S.-H.K.) used the ballpoint- pen technique to measure the diameters of the erythemas and indurations.

Surrogate marker for residual immunity to smallpox. After measuring the reactions to the intradermal skin test, we vaccinated all subjects with either 1:1- or 1:10-diluted Lancy-Vaxina, as scheduled in the smallpox-vaccination trial, and examined them on days 5–6, 8, 10–12, and 13–14 after vaccination. We defined the absence of residual immunity to smallpox as the development of a typical primary response at the site of vaccination—that is, the presence of clearly identifiable pustules 8 days after smallpox vaccination [11]; we defined the presence of residual immunity to smallpox as the development of a typical revaccinee’s response—that is, a rapidly evolving area of definite induration or congestion surrounding a central lesion consisting of a scab or ulcer 8 days after smallpox vaccination [11]; if we had difficulty in clearly distinguishing between “typical primary response” and “typical revaccinee’s response,” we classified the case as an “indeterminate response.” Furthermore, we used additional continuous dependent variables for evaluation of residual immunity to smallpox, such as the size of vaccination-site induration at each day after smallpox vaccination and the time to scab formation, because the presence of variable degrees of residual immunity in previously vaccinated individuals can produce a spectrum of reactions.

Neutralizing-antibody assays. Serum samples were collected just before the intradermal skin test was performed and were assayed as described elsewhere [3, 14]. In brief, 50 μL of duplicate 2-fold dilutions of serum were mixed, at a 1:1 ratio, with 30–50 pfu of vaccinia-virus suspension (fresh vaccinia virus obtained from lyophilized Lancy-Vaxina) and then were incubated for 2 h at 37°C. After incubation, test samples and control samples were added onto monolayers of BSC-40 cells in 24-well plates. Adsorption, incubation, fixation, and staining were performed according to the protocol described above. The plaque-reduction–neutralizing titer (PRNT_{50}) was defined as the serum dilution that yielded a 50% reduction in vaccinia plaque-forming units.

ELISPOT assay. Cell-mediated immune responses to smallpox vaccination were considered to be the immediate vaccinia-specific IFN-γ–producing T cell response, as determined by use of the ELISPOT assay. The assay was modified from the method that has been described elsewhere [1, 15]. In brief, ~60 mL of venous blood was obtained from each subject just before the intradermal skin test. Within 6 h of collection, peripheral-blood mononuclear cells (PBMCs) were isolated by use of Ficoll-Hypaque density gradients. The PBMCs were then resuspended, at a concentration of 10^7 cells/mL, in RPMI–20% fetal bovine

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serum (FBS)–10% dimethyl sulfoxide and were cryopreserved. The cryopreserved PBMCs were thawed and then were washed once with RPMI 1640 medium supplemented with 10% FBS and 50 U/mL Benzonase (Sigma-Aldrich). Cells were again washed and then were resuspended, at a concentration of $5 \times 10^5$ cells/mL, with RPMI 1640 supplemented with 10% FBS. The prepared PBMCs were infected, for 1 h, with live vaccinia virus (fresh vaccinia virus obtained from lyophilized Lancy-Vaxina) at an MOI of 1. Cells were washed and then were added to 96-well ELISPOT plates (BD Biosciences Pharmingen) coated with anti–human IFN-γ antibody (BD ELISPOT Human IFN-γ Kit). Uninfected cells with medium alone were used as negative controls, and uninfected PBMCs stimulated with purified PHA (Sigma-Aldrich) were used as positive controls. Cells were cultured, at a concentration of $5 \times 10^5$ cells/well, in duplicate wells, for 18 h at 37°C. Spots were counted by use of an automated microscope (Carl Zeiss MicroImaging) after the background value, obtained by use of unstimulated cells, was subtracted.

**Statistical analysis.** Statistical analyses were performed by use of the SPSS for Windows (version 10.0; SPSS) software package. Kruskal-Wallis 1-way analysis of variance was used to compare the differences between groups. Linear correlation was evaluated by use of Pearson’s correlation coefficient ($r$). Diagnostic performance was expressed in terms of sensitivity, specificity, positive and negative predictive values, and the area under receiver operating characteristics (ROC) curves. All tests of significance were 2-tailed; $P \leq 0.05$ was considered to be significant.

**RESULTS**

**Baseline clinical characteristics and skin reactions after smallpox vaccination.** The results of the smallpox-vaccine–dilution trial have been published elsewhere [10]. Of the 112 subjects in that trial, only 83 (74%)—63 with and 20 without a history of vaccinia-virus vaccination—agreed to participate in the intradermal skin test. Inactivated vaccinia virus was intradermally inoculated, and the sizes of the skin reactions were measured 48 h later. Immediately after that measurement had been made, the subjects were immunized with smallpox vaccine, for the vaccine-dilution trial. Of the 83 subjects, 32 (39%) received undiluted vaccine, and 51 (61%) received a 1:10 vaccine dilution (for further details, see footnotes b and c to table 1). All 83 subjects showed a “take” reaction after smallpox vaccination—30 (36%) had the typical primary response, 34 (41%) had the typical revaccinee’s response, and 19 (23%) had an indeterminate response. Baseline clinical characteristics are shown in table 1. Before smallpox vaccination of the 83 subjects, we obtained serum samples from all 83 (100%) of them and PBMC samples from 52 (63%) of them and evaluated these samples for humoral immune response and cell-mediated immune response, respectively.

**Results of the intradermal skin test—and relationship between the intradermal skin test and in vitro assays for humoral and cell-mediated immunity.** Except for local itching in a few subjects, there were no adverse reactions to the intradermal skin test with inactivated vaccinia virus; no subjects developed skin reactions to the control solution. The results of the intradermal skin test, as based on skin reactions to smallpox vaccination, are shown in figure 1. After smallpox vaccination, the mean ± SD size of induration was 1.7 ± 1.0 mm in subjects with the typical primary response, 4.2 ± 2.3 mm in those with an indeterminate response, and 6.1 ± 2.6 mm in those with the typical revaccinee’s response ($P < 0.001$); the mean ± SD size of erythema was 3.4 ± 5.3 mm in subjects with the typical primary response, 10.1 ± 7.7 mm in those with an indeterminate response, and 18.2 ± 9.3 mm in those with the typical revaccinee’s response ($P < 0.001$). The relationship between T cell responses generating vaccinia-specific IFN-γ and skin reactions to smallpox vaccination is shown in figure 2.

There was a significant correlation between neutralizing-antibody titer and both size of induration after the intradermal skin test ($r = 0.49; P < 0.001$) and size of erythema after the intradermal skin test ($r = 0.51; P < 0.001$), whereas there was no significant correlation between vaccinia-specific IFN-γ-producing T cell response and either size of induration after the intradermal skin test ($r = 0.22; P = 0.13$) or size of erythema after the intradermal skin test ($r = 0.17; P = 0.21$).

**Diagnostic performance of parameters for prediction of residual immunity to smallpox.** To estimate the diagnostic performances of various parameters for prediction of residual immunity to smallpox, we used skin reactions to smallpox vaccination as a surrogate marker for residual immunity to smallpox; that is, of the 83 subjects, 30 (36%) with the typical primary response were classified as having residual immunity, and the remaining 19 (23%) with an indeterminate response were classified as having no residual immunity to smallpox, 34 (41%) with the typical revaccinee’s response were classified as having residual immunity, and the remaining 19 (23%) with an indeterminate response were included in the analysis. Because there are many possible cutoff values for prediction of residual immunity to smallpox, figure 3 shows ROC curves of the various parameters for it. Each ROC curve reveals that the optimal trade-off inflection points for induration size after the intradermal skin test, erythema size after the intradermal skin test, neutralizing-antibody titer, and vaccinia-specific IFN-γ-producing T cell response were 4 mm, 8 mm, $\geq 1:8$, and $\geq 9$ spot-forming cells (SFU)/10⁶ PBMCs, respectively. The statistics for each cutoff value for prediction of residual immunity to smallpox are shown in table 2. As shown in figure 3, the induration size after the intradermal skin
Correlations between parameters for prediction of residual immunity to smallpox and continuous dependent variables for evaluation of it. In addition to the dichotomous classification of residual immunity to smallpox, we also compared the parameters for prediction of residual immunity and continuous dependent variables for evaluation of residual immunity to smallpox, such as the size of vaccination-site induration at each day after smallpox vaccination and the time to scab formation (table 3). Both size of induration at 13–14 days after smallpox vaccination and time to scab formation were significantly correlated with size of induration after the intradermal skin test \( r = 0.59 \) and \( r = 0.65 \), respectively; \( P < .001 \), size of erythema after the intradermal skin test \( r = 0.51 \) and \( r = 0.62 \), respectively; \( P < .001 \), and neutralizing-antibody titer \( r = 0.41 \) and \( r = 0.45 \), respectively; \( P < .001 \).

**DISCUSSION**

Residual immune response decades after smallpox vaccination is an important issue for public health and vaccine development. Recent in vitro studies have shown that vaccinia-specific memory B cells persist for >50 years and that >90% of subjects vaccinated 25–75 years ago still have substantial humoral and cell-mediated immunity [2, 3]. On the other hand, Combadiere et al. [1] have reported that only 20% of subjects vaccinated 13–25 years ago display IFN-γ–producing effector-memory responses. Also, Hsieh et al. have demonstrated that "T cell reactivity against vaccinia virus ... start[s] to wane within 20–30 years after smallpox vaccination" [4, p. 86] and is "as low as those in unvaccinated subjects" [4, p. 89]. These inconsistent results may be due to the fact that the methods for evaluation of residual immunity are complex and not standardized. The present study has 2 unique features relevant to this issue: the first is that the method that we developed for evaluation of residual immunity to smallpox is simpler and more reliable than that which uses in vitro immunologic parameters such as neutralizing antibody or IFN-γ–producing effector-memory responses; the second is that we used a unique human model of poxvirus infection to evaluate how these parameters correlate with residual immunity to smallpox. Although the portals of entry for variola virus and vaccinia virus are different, the skin lesions resulting from intradermal inoculation with vaccinia virus are similar, in both development and pathogenesis, to
those resulting from smallpox [16, 17]. Therefore, we think that inoculation with vaccinia virus can be regarded as a human model of poxvirus infection.

Corroborating what has also been described in the historical literature regarding smallpox [18, 19], the present study has demonstrated that there is a correlation between the in vivo intradermal skin test and humoral immunity. However, higher levels of neutralizing antibodies may simply be associated with higher underlying T cell immunity. In addition, humoral and cell-mediated immune mechanisms work interdependently in controlling vaccinia-virus infection in humans, as has been shown in previous studies [20, 21]. Hence, we believe that both humoral and cell-mediated immunity make significant contributions to protection against smallpox. In contrast, we did not find that residual cell-mediated immunity as assessed by in vitro ELISPOT assay was correlated with either the outcome of in vivo intradermal skin tests or skin reactions after smallpox vaccination. One possible explanation for this discrepancy could be that the ELISPOT assay was neither sensitive enough nor specific enough to detect the presence of latent memory vaccinia-virus–specific CD8+ T cells before revaccination [1]. For detection of these extremely low frequencies of vaccinia-specific T cells, a proliferation assay (of either proliferative or central memory T cells) or intracellular IFN-γ staining might be more appropriate than is the ELISPOT assay (of immediate effector-memory T cells) [1]. Therefore, further studies are necessary to compare different assays for cell-mediated immunity, involving stimulation with various strains of vaccinia virus, with either delayed-type hypersensitivity reactions after an intradermal inoculation with inactivated vaccinia virus or skin reactions after smallpox vaccination.

In the present study, we have proposed that the existence of residual immunity to vaccinia virus (and, by implication, to variola virus) can be assessed by a method of intradermal inoculation with live vaccinia virus. Indeed, residual immunity to smallpox does not mean protection from smallpox infection, because resistance to intradermal inoculation with vaccinia virus may not be equivalent to resistance to variola virus [22]. Instead, residual immunity implies the ability to suppress the replication of an orthopoxvirus; hence, residual immunity to smallpox may reduce the severity (i.e., mortality) of a smallpox infection. In addition, we have demonstrated that such immunity can be predicted by the safer and simpler method of evaluating the response to inactivated vaccinia virus. We have observed that a response consistent with a delayed-type hypersensitivity reaction to inactivated vaccine is strongly correlated with the occurrence of the "typical revaccinee’s response," indicating that the intradermal skin test reliably predicts the ability to suppress the replication of an orthopoxvirus.

The availability of newer types of vaccine that could be used reduces the importance of one of the issues related to evaluation of residual immunity to smallpox; however, such evaluation is the only method that can be used to estimate how long, in the human model, the memory response of humoral or cell-mediated immunity persists without antigenic stimulation, because vaccinia virus does not cause a chronic or latent infection in humans and because there is no possibility of reexposure to it [23]. In addition, the recent study by Stittelaar et al. indicates that, for treatment of individuals who may have been exposed to smallpox in an outbreak situation, cidofovir should be more effective than smallpox vaccination [24]. We believe that the results of the present study will be of help in the development of a nationwide preparedness for smallpox infection, by relating antiviral treatment and vaccination priorities to the available stocks of antiviral agents or vaccines. Thus, when stocks of antiviral agents are insufficient for treatment of all individuals who may have been exposed to smallpox, they first should be given to vaccinia-naïve individuals (i.e., those without residual immunity to smallpox), who might have a more severe course of smallpox disease and a more complicated course after smallpox vaccination than would previously vaccinated individuals (i.e., those with residual immunity to smallpox), and the remaining stock of smallpox vaccine should then be given to the previously vaccinated individuals. Indeed, the intradermal skin test with inactivated vaccinia virus has already been used to differentiate between subjects with an uncomplicated smallpox vaccination and those in whom the lesion will develop into progressive vaccinia [20, 21].

The present study has several limitations. First, variability
Figure 2. Dot plot showing distribution of immediate vaccinia-specific interferon-γ (IFN-γ)–producing T cell responses assessed by ELISPOT assay, in relation to skin reactions after smallpox vaccination. The horizontal lines denote the mean values for the groups. ANOVA, analysis of variance.

Figure 3. Receiver operating characteristics (ROC) curves of various parameters for prediction of residual immunity to smallpox. Of the 83 subjects, the 64 who had either the typical primary response ($n = 30$) or the typical revaccinee’s response ($n = 34$) were included in the analyses; the remaining 19 subjects, who had an indeterminate response, were excluded. Of these 64 subjects, 38 from whom peripheral-blood mononuclear cells were available for ELISPOT assay were included in the ROC curve for the parameter of vaccinia-specific interferon-γ (IFN-γ)–producing T cell response.
was examined in a blinded fashion, and (2) those instances in which it was difficult to make a clear distinction were classified as indeterminate responses and therefore were excluded from the final analysis of diagnostic performance. Furthermore, we used additional continuous dependent variables—namely, the size of induration at the vaccination site at each day after smallpox vaccination and the time to scab formation—to evaluate residual immunity to smallpox. The similarity in the results of the 2 analyses suggests that a misclassification bias with regard to the vaccination response is unlikely. Third, some may argue that the vaccine dilution itself may affect the skin reactions after smallpox vaccination; however, many published studies of the first-generation smallpox vaccine have shown that, up to a certain level, dilution of vaccine does not affect skin reactivity [10, 25–27], and hence we would assume that, once viral replication in the skin is initiated, the resulting lesion is similar, regardless of the dose of vaccine. To our knowledge, only 1 published article [28] has reported (albeit without any supporting assay of viral kinetics) that diluted vaccinia virus increases local viral replication, which results in increased local inflammatory response; in fact, a previous Lancy-Vaxina trial [10] revealed that neither the size of skin lesions nor quantitative local viral replication differed between a group of 1:1-diluted-vaccine recipients and a group of 1:10-diluted-vaccine recipients, a finding that tends to counter the suggestion that, compared with undiluted vaccine, 1:10-diluted vaccine is more reactogenic or may induce more delayed cutaneous response. Also, we did not find any vaccine-dilution-dependent differences between skin reactions after smallpox vaccination of vaccinia-naïve subjects and those of previously vaccinated subjects (table 1). Finally, caution should be used in assessing the results of intradermal skin tests in individuals who are at the extremes of age, in those who are immunocompromised, and in those with atopic dermatitis—a consideration that may limit the generalizability of the findings of the present study.

In conclusion, the results of the present study suggest that an intradermal skin test using inactivated vaccinia virus may be a simple, rapidly interpretable, and reliable method for prediction of residual immunity to smallpox. Such a test could be

### Table 2. Operating characteristics for residual immunity to smallpox.

<table>
<thead>
<tr>
<th>Test result</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intradermal skin test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>When induration size is ( \geq 4 ) mm(^a)</td>
<td>85 (69–95)</td>
<td>97 (83–100)</td>
<td>97 (83–100)</td>
</tr>
<tr>
<td>When erythema size is ( \geq 8 ) mm(^a)</td>
<td>91 (76–98)</td>
<td>90 (74–98)</td>
<td>91 (76–98)</td>
</tr>
<tr>
<td>Humoral immunity, when neutralizing-antibody titer is ( \geq 1:8 )(^a)</td>
<td>79 (62–91)</td>
<td>80 (61–92)</td>
<td>82 (65–93)</td>
</tr>
<tr>
<td>Vaccinia-specific IFN-γ-producing T cell response ( \geq 9 ) sfc/10(^6) PBMCs(^b)</td>
<td>32 (14–55)</td>
<td>63 (35–85)</td>
<td>54 (25–81)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are % (95% confidence interval). Cutoff values for parameters are based on the ROC curves shown in figure 2. IFN, interferon; PBMCs, peripheral-blood mononuclear cells; ROC, receiver operating characteristics; sfc, spot-forming cells.

\(^a\) Of the 83 subjects, the 64 who had either the typical primary response (\( n = 30 \)) or the typical revaccine’s response (\( n = 34 \)) were included in the analyses; the remaining 19 subjects, who had an indeterminate response, were excluded.

\(^b\) The 38 subjects from whom peripheral-blood mononuclear cells were available for ELISPOT assay were included in the analyses.

### Table 3. Correlations between parameters for prediction of residual immunity to smallpox and continuous dependent variables for evaluation of it.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Size of induration after smallpox vaccination</th>
<th>Time to scab formation</th>
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<tbody>
<tr>
<td></td>
<td>5–6 Days</td>
<td>8 Days</td>
</tr>
<tr>
<td>Size of induration after intradermal skin test(^a)</td>
<td>.17 (.12)</td>
<td>.12 (.28)</td>
</tr>
<tr>
<td>Size of erythema after intradermal skin test(^a)</td>
<td>.11 (.32)</td>
<td>.17 (.13)</td>
</tr>
<tr>
<td>Neutralizing-antibody titer(^a)</td>
<td>.01 (.92)</td>
<td>.05 (.64)</td>
</tr>
<tr>
<td>Vaccinia-specific IFN-γ-producing T cell response(^b)</td>
<td>.11 (.42)</td>
<td>.15 (.30)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are Pearson’s correlation coefficient \( r \) (\( P \) value). IFN, interferon.

\(^a\) All 83 subjects were included in the analysis.

\(^b\) The 52 subjects from whom peripheral-blood mononuclear cells were available for ELISPOT assay were included in the analysis.
useful for evaluation of immunity to smallpox, in individuals and in populations.

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References