Alternative Immunological Markers to Document Successful Multiple Smallpox Revaccinations

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Background. Successful smallpox vaccination is traditionally defined by clinical response ("take"). Nevertheless, only 60% of subjects in the 2002 Israeli smallpox revaccination campaign developed clinical take. More sensitive immunological markers are needed to document successful revaccination. We compared the level of vaccinia-specific immune markers among subjects who did or did not develop clinical take following revaccination.

Methods. Forty subjects who participated in 2002 smallpox revaccination campaign and developed clinical take were individually matched for age, sex, and smallpox vaccinations with subjects who did not develop clinical take ("no-take"). Vaccinia immunity markers were examined prior to and 14 days and 2 years after revaccination.

Results. Higher levels of total immunoglobulin (Ig) G, IgG1, and neutralizing antibodies (highest dilution of serum that inhibited the cytopathic effect by at least 50% [PRNT50]) were observed in the no-take group before vaccination (166 vs 94.3 ELISAU/mL [P < .05], 53.2 vs 34.5 ELISAU/mL [P < .05], and 30% vs 19.7% [P < .05], respectively). The mean time since last smallpox vaccination was longer in the take group than in the no-take group. Total IgG, IgG subclasses, avidity index, and PRNT50 levels were higher among "take" than "no-take" volunteers 14 days after revaccination. The no-take group was not inferior to the take group in all vaccinia immune marker levels measured 24 months after vaccination. Moreover, mean interferon-γ secretion and the percentage of serum samples with PRNT50 levels >1:32 were significantly higher in the No-take group than in the take group (677.5 vs 282.7 pg/mL [P < .05] and 95% vs 80% [P < .05], respectively).

Conclusions. The overwhelming majority of subjects in the no-take group were successfully revaccinated against smallpox. Under circumstances of emergent smallpox mass immunization, there is no need for revaccination success assessment among individuals who have received multiple previous smallpox vaccinations.

Smallpox was declared eradicated in 1980 after a successful vaccination campaign using the live-attenuated vaccinia vaccine [1]. The threat of intentional smallpox dissemination emerging following the rise of global terror led various countries to reassess their population immune status to smallpox and to prepare strategic plans for mass vaccination; some such countries even immunized a group of potential first responders [2–5].

Traditional smallpox vaccine was produced from exudates obtained after infecting animal skin or chorioalantoic membrane of chicken embryos with vaccinia virus. The vaccine was administered through scarification of the skin using needles, bifurcated needles, rotary lancets, or jet injectors. The classical criterion for successful vaccination against smallpox was the "clinical take," defined as the appearance of a vesicle or pustule at the site of the vaccination 7–9 days after immunization. Surgical and bifurcated needles are probably of equal efficiency in achieving a successful vaccination [6, 7].

Epidemiological studies of smallpox outbreaks suggested that smallpox vaccination provided decades or even life-long protection against lethal disease for its recipients [8]. Accordingly, several studies found presence of a wide range of humoral and cellular immunity markers to vaccinia that persist for decades after smallpox vaccination. Among these markers were vaccinia-specific total immunoglobulin (Ig) G [9, 10], neutralizing antibodies [6], memory B cells [11], and memory T cells [10–14]. Except in 2 studies on...
vaccinia-neutralizing antibodies [15, 16], most of these immune markers were never correlated with protective immunity to smallpox.

In the 2002 smallpox revaccination campaign in Israel, only 60% of subjects developed clinical take [17]. These findings are compatible with clinical take percentages after revaccination reported earlier (56%–82%) [18, 19]. We hypothesized that monitoring of clinical take might be not sensitive enough in case of revaccination. Alternative, more sensitive immunological markers are needed to document successful revaccination. To test this hypothesis, we compared the level of various vaccinia-specific immune markers among subjects who did or did not develop clinical take after revaccination.

**METHODS**

**Study Design and Population**

We conducted a historical prospective study in a cohort of 159 healthy Israeli adults (age, 24–52 years) who participated in a smallpox revaccination campaign of first responders in the Israeli Defense Forces during the winter of 2002–2003 [17]. All subjects were inoculated with vaccinia virus, Lister strain, using the multiple-puncture technique. We compared the presence and amount of various markers of immunity to smallpox between subjects who did not experience clinical take (the “no-take” group) and subjects who did experience clinical take (the “take” group).

Complete information on medical history, prior smallpox vaccinations, and a detailed, well-documented follow-up on clinical and immunological reactions of participants was available. The safety, immunogenicity, and reactivity of the vaccine in this group were recently reported [17].

Of 159 subjects who participated in the Israeli Defense Forces revaccination campaign study, 100 subjects agreed to extend the follow-up period. After matching for age (±3 years), sex, and number of prior smallpox vaccinations (±1 vaccination), 80 subjects (40 in each group) were included in the final analysis.

**Laboratory Procedures**

**Vaccine Production and Administration.** The smallpox vaccine used was manufactured by the Central Laboratories of the Israeli Ministry of Health. Vaccine was produced by inoculation of embryonated chicken eggs with the Lister strain of vaccinia virus. After an incubation period, the virus was harvested from the chorioallantoic membranes of the chicken embryos. Each vaccine vial contained a suspension of $10^7$ pock-forming units per milliliter (PFU/mL) of vaccinia virus.

The subjects were inoculated by the application of 20 mL of the vaccine suspension ($10^7$ PFU/mL) on the skin overlying the deltoid muscle. A standard 23-gauge disposable syringe needle was then used to introduce the vaccine intradermally via 15 brisk punctures within a 2–3-mm cluster. The vaccination site was then covered with semi-permeable adhesive dressing.

**Definition of Clinical Take.** Clinical reactions in vaccinees were actively monitored 3 times during the study by using clinical questionnaires and interviews on days 4 (range, days 3–4), 7 (range, days 7–9), and 14 (range, days 14–18) after vaccination. A successful vaccination (ie, one in which clinical take occurred) was defined by the presence of vesicle, pustule, ulcer, or scab 7–9 days after vaccination [17]. Lesion diameter was not part of the criteria for clinical take evaluation. However, subjects with positive take had a lesion diameter range of 3–200 mm, compared with 0–15 mm in the no-take group [17].

**Cell and Serum Separation.** Serum samples (10 mL) and blood samples (40 mL) were obtained using EDTA tubes (BD). Peripheral blood mononuclear cells (PBMCs) were separated from fresh blood using Vacutainer cell preparation tubes (BD) and were then resuspended in R-10 solution (RPMI 1640 plus 10% fetal calf serum supplemented with penicillin, streptomycin, and L-glutamine). Fresh cells were used in all assays. Serum was separated using serum separation tubes.

**Serum Vaccinia Total IgG and IgG1-3 Subclasses Measurement.** We used an enzyme-linked immunosorbent assay (ELISA), as described elsewhere [17]. In brief, microtiter 96-well plates were coated with 15 μg/mL β-propiolactone–inactivated crude vaccinia antigen (IHD-J strain), 50 μL/well (Omnix Lab. Nes Ziona, Israel) buffered with NaHCO3 buffer (50 mmol/L; pH, 9.6), and then blocked with TSTA buffer (50 mmol/L Tris [pH, 7.6]; 142 mmol/L sodium chloride; 0.05% sodium azide; 0.05% Tween 20; and 2% bovine serum albumin). Serial 2-fold dilutions of the tested serum (100 μL) were incubated in the plates for 2 h at 37°C. Plates were developed with alkaline phosphatase–conjugated rabbit anti-human IgG followed by p-nitrophenyl phosphate as substrate (both from). Finally, absorbance at 405 nm was measured and calculated to be expressed as ELISAU/mL.

For measurement of levels of vaccinia IgG subclasses 1–3, a specific conjugate was used for each subclass (KPL). Serum specimens were tested at 1/300 and 1/1200 dilutions. Results for IgG subclasses 1–3 were calculated and expressed as ELISAU/mL.

**Total IgG Antibody Avidity Test.** Antibody avidity was measured by an elution ELISA with the chaotrope thiocyanate, as described elsewhere [20], modified for the vaccinia ELISA IgG assay. In brief, serum specimens were diluted to achieve an optical density of ~1.0 and were then incubated on an antigen-coated plate for 1 h at 37°C. The plates were washed, and ammonium thiocyanate diluted in the serum buffer at various concentrations from 0 to 4 M was added to the wells. After 15 min at room temperature, the plates were washed and alkaline phosphatase–conjugated rabbit anti-human IgG, followed by p-nitrophenyl phosphate as substrate, were added. The absorbance was then read at 450 nm. An avidity index was generated by plotting the log of the percent reduction against the thiocyanate concentration and calculating the amount of thiocyanate required to reduce the absorbance in a given serum by 50%.
T Cell Interferon (IFN)-γ Secretion Test. We used the method described by Samandary et al [21], with some modifications. In brief, fresh PBMCs (density, $1.5 \times 10^5$ cells/mL) were resuspended in AIM-V medium (Invitrogen) together with 5 μL/mL of crude, inactivated vaccinia antigen (IHD-J strain, β-propiolactone–inactivated, $10^7$ PFU/mL; Omrix Lab.) for 72 hours in 5% CO₂ and 37°C humidified chamber (Forma Scientific). Phytohemagglutinin A (PHA) and AIM-V alone were used as positive and negative controls. Supernatant was collected and evaluated for IFN-γ concentrations using an ELISA commercial kit (R&D Systems). The amount of IFNγ secreted was determined by subtracting the negative control value from the average level. The lower limit of detection was 7.8 pg/mL.

Vaccinia Specific Memory B Cell Assay. This method was adopted from Crotty et al [22], with minor variations. In brief, PBMCs were plated in 24-well dishes at 5 $\times 10^5$ cells/well in RPMI 1640 supplemented with 0.1% bovine serum albumin (Sigma-Aldrich), 4% HEPES, 0.1% nonessential amino acids (Gibco), 10 μL/mL of crude, inactivated vaccinia antigen (IHD-J strain, β-propiolactone–inactivated; 107 PFU/mL; Omrix Lab.). To detect all IgG-secreting cells, a separate plate was coated with 10 μL/mL of 100 TCD50 live vaccinia virus. Neutrophilic granulocytes (Sigma-Aldrich) were added to visualize the spots, which were further counted using a BioSys 4000 ELISPOT reader. Results are expressed as percentage of vaccinia virus–specific B cells of the total memory B cells.

Vaccinia Neutralizing Antibodies Assay. We used the method previously described [23]. In brief, 100-μL samples of 2-fold dilutions of serum were mixed with 100 μL of 100 TCD50 (50% tissue culture infective dose) vaccinia virus (IHD-J strain). Residual vaccinia virus was detected on Vero cells. Neutralization titers were defined as the highest dilution of serum that inhibited the cytopathic effect by at least 50% (PRNT50).

Data Validation. The reproducibility of the assays was documented by the retesting of 10% of samples. For all the assays use, the coefficient of variance was lower than 15%. Blood samples obtained from 9 vaccinia-naive subjects were used to confirm the specificity of the immunological markers. The vaccinia-naive group included in this study had a mean age (± standard deviation [SD]) of 20.3 ± 1 years, and 66% were women.

No prior laboratory data were used for analysis in this study. Tests were designed so that matched samples were examined in the same procedure.

Statistical Analysis

Statistical analysis was performed using SPSS software, version 13 (SPSS Technologies). Subject matching was performed using SAS software, version 8 (SAS Institute) using a described matching algorithm [24]. Comparisons of continuous variables between the study groups were performed using paired t test. The Wilcoxon rank test was used for nonparametric variables. Differences in categorical variables were compared using the χ² test. Nonparametric multiple group comparisons were performed using the Friedman and Dunn post test.

RESULTS

Study Sample

Baseline characteristics of study subjects are summarized in table 1. The mean ages (±SD) of the take and no-take groups were 34 ± 7.5 and 33 ± 6.9 years, respectively. Sixty-eight percent of the subjects were men. The most frequent number of previous vaccinations was 3 in both groups. All of the vaccinees who participated in the study were examined 24 months after revaccination. The mean number of years (±SD) that had passed from the last smallpox vaccination before revaccination was higher in the take group than in the no-take group (18.7 ± 7.2 years vs. 15.6 ± 6.5 years, P < .05).

Table 1. Baseline Characteristics of Study Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>The no-take group (n = 40)</th>
<th>The take group (n = 40)</th>
<th>Total population (n = 159)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years ± SD</td>
<td>33 ± 6.9</td>
<td>34 ± 7.5</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>No. (%) of male subjects</td>
<td>23 (68)</td>
<td>23 (68)</td>
<td>102 (60)</td>
</tr>
<tr>
<td>Median no. of prior vaccinations (range)</td>
<td>3 (1–4)</td>
<td>3 (1–3)</td>
<td>3 (1–4)</td>
</tr>
<tr>
<td>Interval since prior vaccination, mean years ± SD</td>
<td>16 ± 6.5</td>
<td>19 ± 7.2</td>
<td>20 ± 5.5</td>
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</tbody>
</table>

NOTE. SD, standard deviation.

* Examined in the present study.

Revaccinated against smallpox and previously investigated [17].
Serum Total IgG and IgG Subclasses Levels
Table 2 summarizes serum total IgG levels in take and no-take groups. Total IgG levels were lower in the no-take group than in the take group at day 14 (286.2 vs 286.1 ELISAU/mL; \( P = .001 \)). In contrast, total IgG levels at day 0 were higher in no-take group than in take group (166 vs 94.3 ELISAU/mL; \( P = .05 \)). IgG levels were similar between the groups 24 months after the vaccination (145.3 vs 214 ELISAU/mL; \( P = .126 \)). IgG levels at day 0 were negatively correlated with the number of years that had passed since the last vaccination, after adjustment for sex, age, and prior number of vaccinations (\( r^2 = -0.273; P = .013 \)).

Levels of IgG1 subclass were higher in no-take group than in the take group on day 0 (53.2 vs 34.5 ELISAU/mL; \( P = .05 \)). Levels of all IgG subclasses (1–3) were found to be lower in no-take group than in the take group on day 14 (134.7 vs 1016 ELISAU/mL; \( P = .001 \), 153 vs 239.5 ELISAU/mL; \( P = .009 \), and 60.3 vs 123.7 ELISAU/mL; \( P = .01 \), respectively). Serum levels of both total IgG and the IgG subclasses were undetectable in the naive subjects.

Neutralization Assay
The mean PRNT50 levels in no-take group, compared with the take group, were higher on day 0 (30 vs 19.7; \( P = .02 \)) and lower on day 14 (224.1 vs 367; \( P = .03 \)). No significant differences were found between the groups 24 months after vaccination. The PRNT50 levels were all \( 1:4 \) in naive subjects. The measurement units are PRNT50 for neutralization assay.

### Table 2. Vaccinia Immune Marker Levels in the “Take” versus “No-Take” Groups

<table>
<thead>
<tr>
<th>Marker, time</th>
<th>The no-take group</th>
<th>The take group</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG level, mean ELISAU/mL (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>166.0 (94–238)</td>
<td>94.3 (63.6–115)</td>
<td>.05</td>
</tr>
<tr>
<td>Day 14</td>
<td>286.1 (166.5–405.8)</td>
<td>286.2 (1724–4001)</td>
<td>.001</td>
</tr>
<tr>
<td>Month 24</td>
<td>145.3 (114–176.7)</td>
<td>214.2 (137.1–291.2)</td>
<td>.12</td>
</tr>
<tr>
<td>Total IgG1 level, mean ELISAU/mL (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>53.2 (33.3–73)</td>
<td>34.5 (23.8–45.3)</td>
<td>.05</td>
</tr>
<tr>
<td>Day 14</td>
<td>134.7 (68.6–201)</td>
<td>1016 (616.4–1415)</td>
<td>.001</td>
</tr>
<tr>
<td>Month 24</td>
<td>70.3 (51.1–89.6)</td>
<td>103.9 (54.2–153.6)</td>
<td>.23</td>
</tr>
<tr>
<td>IgG2 level, mean ELISAU/mL (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>145.7 (124–167.4)</td>
<td>190 (143.8–236.1)</td>
<td>.08</td>
</tr>
<tr>
<td>Day 14</td>
<td>153 (131–175.1)</td>
<td>239.5 (180.4–298.5)</td>
<td>.009</td>
</tr>
<tr>
<td>Month 24</td>
<td>163 (140.1–186)</td>
<td>209.2 (159.4–259.1)</td>
<td>.08</td>
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<tr>
<td>IgG3 level, mean ELISAU/mL (95% CI)</td>
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<tr>
<td>Day 0</td>
<td>39.6 (28.7–50.6)</td>
<td>33.8 (24–43.5)</td>
<td>.34</td>
</tr>
<tr>
<td>Day 14</td>
<td>60.3 (44–76.8)</td>
<td>123.7 (79.6–167.7)</td>
<td>.01</td>
</tr>
<tr>
<td>Month 24</td>
<td>54.2 (39–69.5)</td>
<td>47.5 (35.4–59.7)</td>
<td>.35</td>
</tr>
<tr>
<td>Mean PRNT50 level (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>30 (23.3–36.7)</td>
<td>19.7 (14.5–24.9)</td>
<td>.02</td>
</tr>
<tr>
<td>Day 14</td>
<td>224.1 (72.2–376)</td>
<td>367 (195–539)</td>
<td>.03</td>
</tr>
<tr>
<td>Month 24</td>
<td>159.5 (14.4–305)</td>
<td>96.8 (59.6–134)</td>
<td>.41</td>
</tr>
<tr>
<td>No. (%) of subjects with a PRNT50 level ( \geq 1:32 )</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Day 0</td>
<td>22 (55)</td>
<td>13 (33)</td>
<td>.04</td>
</tr>
<tr>
<td>Day 14</td>
<td>37 (93)</td>
<td>39 (98)</td>
<td>.32</td>
</tr>
<tr>
<td>Month 24</td>
<td>38 (95)</td>
<td>32 (80)</td>
<td>.04</td>
</tr>
<tr>
<td>Avidity index, M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>2 (1.86–2.17)</td>
<td>2.02 (1.84–2.17)</td>
<td>.92</td>
</tr>
<tr>
<td>Day 14</td>
<td>2.08 (1.9–2.27)</td>
<td>2.4 (2.24–2.56)</td>
<td>.016</td>
</tr>
<tr>
<td>Month 24</td>
<td>2.12 (1.92–2.31)</td>
<td>2.21 (2.08–2.35)</td>
<td>.32</td>
</tr>
<tr>
<td>IFN-( \gamma ) level, mean pg/mL (95% CI)</td>
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<td></td>
<td></td>
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<tr>
<td>vaccine-specific B cell percentage, mean (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>677.5 (386.6–968.3)</td>
<td>282.7 (171.8–393.7)</td>
<td>.014</td>
</tr>
<tr>
<td>Day 14</td>
<td>4.77 (3.42–6.12)</td>
<td>3.74 (2.92–4.57)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; Ig, immunoglobulin; IFN-\( \gamma \), interferon; NS, not significant; PRNT50, highest dilution of serum that inhibited the cytopathic effect by at least 50%.

*Comparison was performed using Wilcoxon test.
IFN-γ Secretion
IFN-γ secretion levels after incubation with crude vaccinia antigen were higher in the no-take than in the take group 24 months after revaccination (677.5 vs 282.7 pg/mL; \( P = .014 \)); levels were undetectable in the 9 naive subjects (Figure 1). Lack of IFN-γ secretion was also observed in 3 subjects in the no-take group.

IgG Antibody Avidity
Overall, the avidity index was lower on day 0 than on days 14 and 24 months (\( P < .001 \) and \( P = .01 \), respectively), with no significant difference between day 14 and 24 months following revaccination (\( P = .195 \)). Avidity index levels were higher in the take group than in the no-take group on day 14 (2.44 vs 2.08M; \( P = .016 \)) but were similar at 24 months (2.21 vs 2.12 M; \( P = .585 \)).

Vaccinia-Specific B Cell Percentage
No differences were found between the groups in the percentage of vaccinia-specific B cells measured in blood samples obtained 24 months after revaccination (see Table 2).

DISCUSSION
Successful smallpox vaccination is traditionally defined by clinical response (take). The Centers for Disease Control and Prevention recommend that both first-time vaccinees and nonnaive vaccinees be instructed to return 6–8 days after vaccination for evaluation of clinical take. In the case of no take, revaccination should be conducted. Smallpox revaccination success rates were reported to be 60%–80% [6].

The routine recommendation for clinical evaluation after revaccination and performance of an additional inoculation for 20%–40% of the revaccinees with no take poses a significant logistic constraint, because vaccinees must be seen by a health professional twice within 6–8 days [25]. This could be especially problematic in the context of mass vaccination, when great numbers of vaccinees are expected to be processed within days, and the need to reexamine each and revaccinate so many would severely tap available medical resources [26].

Here and in a previous study [17], it was shown that subjects who did not experience clinical take had received their previous smallpox vaccination more recently and also had higher levels of vaccinia total IgG and PRNT50 before revaccination, compared with subjects who did experience clinical take. These data suggested that higher persistent and/or residual immunity against vaccinia might be associated in a dose-dependent fashion with a lower rate of clinical take, and therefore, clinical take would not be sensitive enough to document an immune response after revaccination as accepted for primary vaccination. In the present study, we compared the level of various vaccinia-specific immune markers among subjects who did or did not develop clinical take after revaccination. Our assumption was that, 24 months after revaccination, the values of these markers will be similar in the 2 groups, implying that a result of no-take immediately after revaccination would be a false-negative result in reflecting the immune response induced by revaccination.

Indeed, we showed that vaccinia-specific humoral and cellular immune marker levels in the no-take group were similar and even higher than in the take group. IgG levels and avidity indices after 24 months of follow-up were similar in both groups, whereas IFN-γ and PRNT50 levels were significantly higher in the no-take group. Ninety-five percent of the revaccinees without clinical take had levels of PRNT50 antibodies >1:32, 92.5% had detectable levels of IFN-γ, and all had significant levels of either one of the 2 markers 24 months after revaccination. The specificity of these 2 markers was confirmed by their complete absence among the 9 naive subjects also examined in this study.

Interestingly, in a recent study, we showed that previously vaccinated healthy women (mean age ± SD, 49.71 ± 9.1 years) showed detectable levels of IFN-γ and PRNT50 at a mean interval (±SD) of 32.75 ± 10.9 years after vaccination [27]. As predicted, PRNT50 and IFN-γ levels among women were lower than in both study groups 24 months after vaccination.

In a recent study, Treanor et al [28] assessed vaccinia-specific neutralizing antibodies and IFN-γ–producing cells in subjects who had undergone primary vaccination versus those who had undergone revaccination in the recent past (<2 years) and distant past (>10 years). They reported a higher take rate in the distant-past revaccinee group than among recent revaccinees (11 of 15 vs 8 of 15). They also found a lower take rate among both groups of revaccinees, compared with primary vaccinees (73% and 53% vs 100%). Finally, they showed that vaccinia-specific IFN-γ–producing cells and neutralizing antibodies did not correlate strongly with clinical take, suggesting that the response at the local site of inoculation and the parameters measured in the peripheral blood could be considered distinct markers of immunity [28].

Smallpox vaccination is considered protective for decades and even for a lifetime [6, 8, 29]. Eradication period data show that the level of protection from smallpox infection in persons vaccinated in the distant past was between 90.7% and 97.1% [18, 19, 30, 31]. This notion was supported recently by findings of immunologic markers to smallpox in persons who had been vaccinated 60 years earlier [9–11]. However, these surrogate immune markers were not in use during the eradication period; thus, no data are available on their direct relationship to protective immunity against smallpox.

Several studies mentioned a neutralizing antibody titer >1:32 as a potential immune marker for protective immunity to smallpox [10, 15, 16, 32]. Taub et al [32] conducted a longitudinal study in which they quantified vaccinia IgG and neutralizing antibody levels in 209 subjects who had been vaccinated ≥1 time 13–88 years before the evaluation. In 59% of samples, they found that PRNT50 levels ranged from 1:256 to 1:512 and remained tables. Only 3 patients (1.4%) had no measurable neutralizing antibodies.
To examine the effect of number of previous smallpox vaccinations, we compared immune markers in subjects who had undergone 1 versus multiple revaccinations. We found slightly higher immune marker levels (except for PRNT50) in the multiple-revaccination group, but none of the differences were significant. These results are compatible to those of the study by Taub et al [32].

Our study has several limitations. Selection bias is possible, because the study cohort was chosen from among active service army officers (ie, the healthy worker effect). Recall bias regarding number of previous vaccinations was handled by producing an algorithm that extrapolated the number of prior smallpox vaccinations on the basis of public health smallpox vaccination history, date of birth, date of immigration, date of army recruitment, and number of smallpox scars on shoulders and thighs. The evaluation of clinical take was performed in accordance with the Israeli Ministry of Health’s definitions. These definitions are slightly different from the World Health Organization’s definitions; therefore, misclassification bias was possible. This suspicion was refuted after careful comparison between the Israeli Ministry of Health and World Health Organization criteria [17].

In conclusions, we showed that clinical take is not sensitive enough to evaluate smallpox vaccination. Significant levels of specific vaccinia immunological markers, such as PRNT50 and/or IFN-γ, were detected 2 years after revaccination in all no-take subjects. Under the circumstances of emergent smallpox mass immunization, there is no need for revaccination success assessment among individuals who have undergone multiple previous smallpox vaccinations.

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