Evolution of Vaccinia Virus-Specific CD8⁺ Cytotoxic T-Lymphocyte Responses in Primary Vaccinees and Revaccinees

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Determination of successful vaccination with vaccinia virus is based on visual confirmation of a dermal response (take). Some revaccinees do not manifest a take, which may be due to a preexisting immunity rather than to poor technique or inadequate virus. Cytotoxic T-lymphocyte (CTL) response appears to be the most important immune defense in limiting response to vaccination. We evaluated vaccinia virus-specific CTL responses in revaccinees. Subjects with and without takes displayed comparable CTL responses. Vaccinia virus-specific CD8⁺ CTL responses may be useful in interpreting the response to vaccination, particularly in individuals who are revaccinated and have difficult-to-interpret visual takes.

Strategies to protect the public from a bioterrorism threat from smallpox have become a national priority. In the past year, a multiphased vaccination program using vaccinia virus, the related orthopoxvirus, was initiated targeting military and healthcare personnel. While most primary vaccinees have robust responses to the vaccine, up to 10% of revaccinees do not have a classic dermal response, also known as a take, even after repeated vaccinations (3, 4). The absence of a take in an otherwise immunocompetent host is generally thought to represent either faulty technique or an inactivated virus, and repeat vaccination is recommended.

Many revaccinees have an attenuated postvaccination dermal reaction, characterized by induration surrounding a central ulcer that evolves rapidly in comparison to a typical primary reaction. Some of these revaccinees have preexisting neutralizing antibodies against vaccinia virus (5). This accelerated course probably reflects the presence of latent long-lived CD8⁺ memory T-cells that are rapidly recruited to the site of inoculation and that clear virus-infected cells through direct cytolysis or cytokine-mediated cell lysis. Demonstration of preexisting immunity through a vaccinia virus-specific CD8⁺ cytotoxic T-lymphocyte (CTL) enzyme-linked immunospot (ELISPOT) assay may help determine a vaccine take and avoid the need for revaccination. In this report, we describe the magnitude and temporal evolution of vaccinia virus-specific CD8⁺ CTL response in revaccinees with and without a take.

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MATERIALS AND METHODS

Subjects were recruited from the cohort of health care providers who were vaccinated with vaccinia virus as a result of their participation in Rush University Medical Center’s smallpox response team. Volunteers with a history of an immunocompromising condition (e.g., human immunodeficiency virus infection or cancer chemotherapy) or an inflammatory skin disorder or who live with people with the above-named conditions were excluded from participation. A total of 14 health care providers were vaccinated: 13 revaccinees and 1 primary vaccinee. Informed consent was obtained from all subjects.

Subjects were vaccinated at the Chicago Department of Health smallpox vaccination clinic. The vaccine (Dryvax; Wyeth Laboratories, Marietta, Pa.) was provided by the Centers for Disease Control and Prevention (Atlanta, Ga.). Vaccination was performed according to the Centers for Disease Control and Prevention smallpox vaccination method (2). Following vaccination, the vaccination site was covered with a 10- by 13-cm transparent dressing (Op Site; Smith and Nephew Medical Ltd., Hull, England). The dressing was removed, and the vaccination site was examined every 2 days by a member of the hospital’s vaccination site care team. At each dressing change, a photograph of the lesion was taken and stored with the volunteer’s records. The length and width of each induration and lesion were measured using a paper tape measure. A successful vaccine take in a previously vaccinated individual is the formation of a vesicular or pustular lesion or an area of palpable induration or congestion surrounding a central lesion at 6 to 8 days postvaccination which may be a scab or an ulcer (2). Other reactions were considered equivocal.

Vaccinia virus culture. At each dressing change, the dressing was removed and the central part of the vaccination site lesion was swabbed with a Dacron-tipped sterile applicator (Micro Test M4RT; Remel, Kansas City, Mo.) moistened with viral culture medium. The swabs were immediately inoculated into 3 ml of medium (Multi-Microbe Medrano; Micro Test). The medium was frozen and stored at −70°C. Samples were thawed, and the virus was titrated on BSC-1 cells in a plaque assay.

Vaccinia virus plaque assay. Infectious vaccinia virus was assayed as described previously (7) with some modifications. Serial 10-fold dilutions of specimens were prepared in serum-free RPMI medium, and 0.2-ml aliquots were used to inoculate duplicate wells of CV-1 cells in 12-well tissue culture dishes. The specimens, dilutions, and inocula were all vortexed immediately before sampling to keep the virus in suspension. Inoculated cells were incubated at 18°C for 60 min, with redistribution of the inocula every 10 min. The inocula were removed by suction and replaced with 1.5 ml of RPMI containing 8% fetal bovine serum. Cells were incubated for 2 days at 37°C in 5% CO₂ without being disturbed; then, the medium was removed, the cells were fixed with 1 ml of formalin and stained with Coomassie blue, and the plaques were counted. Prior to these experiments, the sensitivity of CV-1 cells to vaccinia virus was compared to that of BSC-1 cells in this plaque assay and the two cell lines were found to be equivalent.

Vaccinia virus-specific gamma interferon ELISPOT assay. Blood samples (20 ml) were obtained prior to vaccination and 4, 7, and 30 days after vaccination and...
collected in heparinized tubes (Becton Dickinson, Franklin Lakes, N.J.). Within 6 h of collection, peripheral blood mononuclear cells (PBMC) were isolated using density centrifugation with lymphocyte separation medium (BioWhittaker, Walkfield, Md.) (lymphocyte separation medium density, 1.077) at 800–1100 g at room temperature for 15 min and resuspended at a concentration of 10^7 cells/ml in 90% fetal bovine serum–10% dimethyl sulfoxide. Cells were frozen immediately using a Mr. Frosty freezing chamber, stored in the chamber at −70°C for 24 to 72 h, and then stored in LN2 vapor phase until thawed.

Our methodology was adapted from that of Ennis et al. (3) with modifications. The same authors have shown that after PBMC stimulation with vaccinia virus, the gamma interferon response was primarily CD8^+ T-cell mediated. The human gamma interferon ELISPOT assay was performed using an ELISPOT kit (BD Pharmingen, San Diego, Calif.) according to the manufacturer’s instructions. Briefly, stock vaccinia virus was added to precoated BD ELISPOT plate wells at a multiplicity of infection of 1 infectious virion/target cell. Cryopreserved PBMC were thawed using the AIDS Clinical Trials Groups protocol (1) and resuspended in R10 medium. Viability of cryopreserved cells ranged from 85 to 95% by trypan blue dye exclusion. PBMC were added to the precoated BD ELISPOT plate wells at a concentration of 2 × 10^5 cells/well, with a final volume of 200 μl/well. Staining was performed after incubating the wells for 20 h at 37°C in a humidified incubator containing 5% CO₂ and 95% air. Cells plus either medium alone or phytohemagglutinin served as a negative or positive control, respectively. Experiments were done in triplicate. Spots were read manually using a dissecting microscope (Bausch & Lomb, Rochester, N.Y.) (10×). The number of gamma interferon-secreting cells was calculated by subtracting the average reading of the negative control wells from the average reading from the three vaccinia virus wells. Results are expressed as number of spots/2 × 10^5 PBMC. A positive result is defined as ≥20 spots/2 × 10^5 PBMC and ≥3 times the negative control result.

Statistical analysis. Differences in parameters between revaccinees who develop a take and those who do not have a take were compared using a Mann-Whitney U-test.

RESULTS

A total of 13 revaccinees, 5 females and 8 males, with ages ranging from 28 to 59 years, were vaccinated. All revaccinees were last vaccinated with vaccinia virus 21 to 50 years earlier. The sole primary vaccinee was a 37-year-old male.

Of 13 revaccinees, 9 had a visual take on days 6 to 8 postvaccination (Fig. 1A). Median induration size was 225 mm² (range, 100 to 1,155 mm²); the induration peaked at a median of 6 days (range, 5 to 13 days) postvaccination and resolved at a median of 12 days (range, 9 to 21 days) postvaccination. One subject was a primary vaccinee who also had a visual take and whose induration peaked at 510 mm² on day 8 postvaccination and resolved by day 21 (Fig. 1C). Four subjects had an attenuated dermal response and were reported as exhibiting equivocal takes (Fig. 1B). Median induration size was 100 mm² (range, 50 to 330 mm²) and peaked at a median of 5.5 days (range, 2 to 8 days) postvaccination; indurations disappeared over a median of 9.5 days (range, 7 to 17 days). Three subjects had indurated areas with central lesions that had resolved by day 6 postvaccination, and one subject had erythema and induration with no central lesion.

A total of 9 of 14 subjects had at least one positive-testing culture from their vaccination sites. Eight were revaccinees. In these subjects, viral titers peaked at a median of 5 days after vaccination and were undetectable by a median of 9 days. In the primary vaccinee, viral cultures from the vaccination site
peak at 9 days and tested negative by day 19. Five subjects had negative-testing cultures at the vaccination site at all time points. Of these five, four were the same subjects that were labeled as exhibiting equivocal takes.

The nine revaccinees with a take had a median vaccinia virus-specific CTL response of 4 spots/2 × 10^5 PBMC at day 4 and a median of 60 spots/2 × 10^5 cells at day 7. Four subjects with neither a positive take nor any cultured virus had comparable responses at 4 days (median = 21 spots/2 × 10^5 PBMC; P = 0.5) and 7 days (median = 83 spots/2 × 10^5 PBMC; P = 0.5) (Fig. 2). Three of the revaccinees with a take had positive CTL responses at baseline (27, 54, and 87 spots/2 × 10^5 cells), while none of the revaccinees without a take did. In both groups, the magnitude of the CTL responses reached a plateau after day 7. In contrast, the magnitude of the CTL responses in the primary vaccinee continued to increase through day 30.

**DISCUSSION**

Protective immunity against smallpox after successful vaccination is probably mediated through cellular mechanisms. The occurrence of a take after vaccination with vaccinia virus correlates with the appearance of vaccinia virus-specific CD8^+ CTL responses and neutralizing antibodies (3, 4, 5). A neutralizing vaccinia virus antibody titer of >1:32 against vaccinia virus was associated with protection of contacts of smallpox victims from disease (8). However, a high antibody level may simply be a marker for robust vaccinia virus-specific T-helper cell function. While adults and children with T-cell immunodeficiency disorders have severe infections after vaccination, children with agammaglobulinemia did not (9). This observation suggests that T-cell-mediated immune responses may be more important in vaccinia virus-specific immunity than B-cell responses. Older reports have estimated that the duration of vaccinia virus-specific immunity wanes over 5 to 10 years. A more recent report, however, demonstrated that 90% of previous vaccinees, some vaccinated more than 70 years earlier, have either detectable vaccinia virus-specific cell-mediated immunity, as determined by intracellular flow cytometry staining of CD4^+ and CD8^+ T-cells for gamma interferon and tumor necrosis factor alpha, or vaccinia virus-specific antibodies (6).

While our sample size is small, we nevertheless demonstrated the presence of vaccinia virus-specific CTL responses in all subjects postvaccination whether or not they had a take. The magnitude of the CTL responses was not statistically different between those with and without a take. This implies that many revaccinees who have either an absent or an equivocal dermal response may not need repeat vaccinations if they are shown to have vaccinia virus-specific CD8^+ T-cell responses. Our study suggests that this determination can be done as early as 7 days postvaccination. Whether one method of assessing CTL response (e.g., intracellular cytokine staining by flow cytometry or tetrameric MHC-1 peptide complexes) is superior to another was not determined in this study, although the ELISPOT assay offers the advantage of technical ease, reproducibility, and the ability to use cryopreserved cells and screen many samples at the same time. This assay, however, was not sensitive enough to detect the presence of latent memory vaccinia virus-specific CD8^+ T-cells prior to revaccination. Intracellular cytokine staining by flow cytometry (6) or cultured ELISPOT assay may detect these cells in revaccinees prior to repeat vaccination.

We observed a higher-than-expected rate of equivocal takes in our cohort compared to the results reported in published studies. Three of the subjects with equivocal takes had been vaccinated at least two other times and had a history of equivocal reactions at each revaccination. Lower rates of take have been previously described for revaccinees with a history of multiple vaccinia virus vaccinations (4; J. R. Romero, C. Allenworth, and A. M. Pour, Abstr. 41st Annu. Meet. Infect. Dis. Soc. Am., San Diego, Calif., abstr. 829, 9 to 12 October, 2003).

These findings are preliminary and need to be confirmed by larger studies that also evaluate the durability of these responses. One limitation of this study was that we did not determine pre- and postvaccination vaccinia virus antibody levels. However, past clinical observations with agammaglobulinemic children have suggested the primacy of T-cell responses over B-cell responses in controlling infection due to vaccinia virus (9). It is possible that the reason for the accelerated dermal responses may be the absence of viable virus in the lesions. However, the increase in magnitude of vaccinia virus-specific CTL responses postvaccination in all subjects with negative cultures suggests that exposure to viral antigens occurred postvaccination.

In summary, we used ELISPOT assays for gamma interferon to demonstrate the presence of vaccinia virus-specific CTL responses in revaccinees who had either positive or equivocal takes. This suggests that this assay may be useful in determining whether revaccinees with no take are candidates for further vaccination. Studies are needed to confirm these findings, to determine whether these vaccinia virus-specific T-cell responses are durable even in those with no visible take, to correlate these results with the antibody response postvaccination, and to discover whether other assays of CD8^+ T-cell function will have more diagnostic sensitivity.
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We do not have a commercial or other association that might pose a conflict of interest.

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