Smallpox: Residual Antibody after Vaccination

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Of all the microorganisms and toxins, poxviruses (Orthopoxvirus) have the greatest potential for use by terrorists. These viruses can spread rapidly through the environment following initial infection. In 1980, the World Health Organization Eradication Program discontinued vaccination for smallpox and declared that the disease had been eliminated. With the threat of smallpox virus as a bioterrorism weapon, questions have been asked about the persistence of protection (as offered by antibodies) following vaccination with vaccinia virus vaccine. To address this, sera from 204 adults vaccinated as children were tested by enzyme immunoassay (EIA) for the presence of vaccinia virus antibody. Of the 204 individuals whose sera were examined for the presence of vaccinia antibody, 165 (80.9%) had been vaccinated once and 39 (19.1%) had been vaccinated at least twice. Of the 165 sera from individuals vaccinated once, 112 (67.9%) were positive. Of the 39 sera from individuals vaccinated more than once, 31 (79.5%) were positive. The presence of a vaccination scar at the time of blood collection was not determined. Fifty-six nonvaccinated individuals, under 30 years of age, were tested by EIA; four of these (7.1%) were positive for vaccinia virus antibody by EIA. Forty-four EIA-positive and 16 EIA-negative sera were also tested by serum neutralization (SN) as a comparison with the EIA test results; one serum (negative by EIA) was SN positive. No attempt was made to ascertain any demographics other than age (date of birth) and “remembered” times of vaccination.

In November 2001, United States citizens became acutely aware of the potential for bioterrorism within our borders. With the introduction of anthrax bacilli (Bacillus anthracis) into the U.S. Postal Service system, the threat of other forms of bioterrorism became a reality. Biological terrorism, or bioterrorism, involves the use or the threat of using disease-spreading microorganisms and/or toxins as weapons of mass destruction. The use of B. anthracis as a weapon of terrorism has resulted in a heightened review of other potential bioterrorism agents. Smallpox virus (variola virus) is considered to pose the greatest risk.

In 1980 the World Health Organization Smallpox Eradication Program declared that the disease, and the need for a vaccine with possible adverse reactions, had been eliminated (1–3). However, stocks of variola virus were “officially” maintained in the United States and in the Soviet Union for experimental purposes (6). The dissolution of the Soviet Union led to concerns regarding safety control of a number of State properties, including stocks of variola virus. Other unknown illicit smallpox virus sources may exist, and these may serve as a potential for bioterrorism threats.

Most adults 35 years old and older were vaccinated for smallpox at least once prior to entering school. An unknown parameter of this smallpox vaccination, however, is the duration of its protection, as determined by the presence of antibody. It is generally accepted that the original vaccination would be protective for about 5 to 10 years, although the World Health Organization Committee on International Quarantine suggested that international travelers be vaccinated within the 3 years prior to travel (5). Multiple vaccinations provide long-term protection (4); however, the duration of antibody from the single original smallpox vaccination has not been recently evaluated. Do adults who received a single vaccination as a child still have any residual antibody? Many individuals are concerned with regard to protection against smallpox infection and specifically with whether they themselves have antibodies. Each serum donor was questioned regarding the number of times he or she was vaccinated.

In this study, the presence of humoral antibody in vaccinated and unvaccinated (control) individuals was investigated. Those who had received additional booster vaccinations were noted, and their results were reported separately from those for individuals who had received a single vaccination. As it is not known whether antibody detected by enzyme immunoassay (EIA) is protective, it was of interest to determine if another antibody test would yield similar results. Accordingly, for comparison, 60 of the EIA-tested sera were also examined by serum neutralization (SN).

MATERIALS AND METHODS

Two hundred four sera from adults of various ages, but at least 35 years of age, were submitted to the ESOTERIX Infectious Disease Center laboratory by their physicians for the detection of vaccinia virus antibody. All claimed to have been vaccinated as children. In the final scoring, results for individuals with more than one vaccination were tabulated separately from those for the group that had been vaccinated only once. In the tabulation of subjects, there were only two criteria: vaccinated once or vaccinated more than once. As there were 39 sera available from individuals vaccinated more than once, these were also tested by EIA. The laboratory received only serum samples; the presence or absence of vaccination scars was not reported.

After blood collection by venipuncture, sera were separated from the clotted blood by centrifugation and then refrigerated at 2 to 8°C. If not tested within 1 week of collection, the sera were frozen at −20°C. Serum titers were not deter-

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mined, as the purpose of this study was to ascertain the number of antibody-positive responses resulting from vaccination.

Approximately half the sera were collected in California, and the other half were collected in Texas. Laboratory personnel performing EIA and SN tests were unaware of the individual’s vaccination status. The majority of test individuals were unable to recall precisely when they received their primary vaccination but thought it was prior to starting kindergarten or elementary school. Fifty-six sera from individuals less than 30 years of age and knowingly never vaccinated were similarly tested by EIA as negative controls.

Variola virus (American Type Culture Collection, VR-156), derived from the New York City Department of Health H9262 strain, was grown and maintained in this laboratory in Vero cells. Following growth, virus preparations were frozen and thawed three times and then centrifuged at 1,500 rpm in a Sorvall refrigerated ultracentrifuge to remove cell debris. The clarified preparation was again centrifuged at 20,000 rpm for 2 h, and the virus pellet was suspended in 2.0 ml of phosphate-buffered saline (PBS), pH 7.2. This test antigen was then titrated by EIA against serially diluted positive serum in a box titration as described below.

Serially diluted antigen (100 μl/well) was added to a 96-well polystyrene EIA plate and incubated for 1 h at room temperature. The plate was then washed three times with PBS containing 0.1% Tween 20 (PBST) and once with PBS. Nonspecific antigen sites were blocked with PBS containing 5% bovine serum albumin (BSA) for 1 h at room temperature. The plate was again washed with PBST and once with PBS. Serially diluted antibody-positive control serum (100 μl) was inoculated into duplicate wells containing the serially diluted antigen. Two wells of each antigen dilution were inoculated per serum dilution. The EIA plate was then incubated for 1 h before each well was washed three times with PBST and once with PBS.

A 1:1,000 dilution of anti-human immunoglobulin G–alkaline phosphatase conjugate (100 μl; Kirkegaard & Perry, Gaithersburg, Md.) was added to each well, and the plate was incubated at room temperature for 1 h. The conjugate dilution was determined by titrating against a serum-antigen box titration in triplicate. The wells were washed three times in PBST and then once with PBS. Paranitrophenyl phosphate substrate (100 μl/well; Kirkegaard & Perry), prepared 10 min prior to use, was added to each well, and the plate was incubated for 15 min at room temperature in the dark. The reaction was stopped with 100 μl of 5% EDTA per well, and results were read at 405 nm with a 630-nm reference filter. The optimum antigen titer and serum dilution were determined to be 1:100. Positive and negative control sera, PBS, and antigen-only wells were included with each test serum.

Based on the results of the box titration, test sera were screened at a 1:100 dilution in PBS containing 3% BSA. The sera were tested in duplicate by using 100 μl of a 1:100 dilution per well of a 1:100 antigen dilution. Concerns that BSA contained cowpox antibody were minimized by the control tests, which contained BSA and were negative.

In addition to the EIA, as a comparison, 60 sera that had been tested by EIA (44 EIA positive and 16 EIA negative) were also examined for SN antibody. After the virus titer in Vero cells (106.0 for 104–105.1 ml) had been determined, 100% tissue culture infective doses of virus was added to a serum dilution of 1:5 in PBS. The virus-serum mixtures were incubated at 37°C for 1 h. Two Vero cell monolayer wells in a 24-well culture plate were inoculated with 0.2 ml of each virus-serum mixture. Test and control (positive and negative) cultures were read at 24 and 48 h.

**RESULTS**

Two hundred four sera were tested by EIA; of these, 165 (80.9%) were from individuals that had been vaccinated once and 39 (19.1%) were from individuals who had been vaccinated more than once. Of the 165 sera from individuals who had been vaccinated once, 112 (67.9%) had vaccinia antibody at the test dilution of 1:100 and 53 (32.1%) were negative. Of 39 sera from subjects that had been vaccinated more than once, 31 (79.5%) were positive. Although the primary purpose of testing these sera was to ascertain if antibody persisted long term after a single vaccination, the results of multiple vaccinations are also reported. Sixty-eight percent of the single-vaccine recipients’ sera were positive for antibody, compared to 79.5% of the sera from subjects with multiple vaccinations.

When the data are arranged according to the decade of birth (Table 1), it is apparent that most of the sera were collected from individuals with birth dates between 1921 and 1970. In each of these decades, a significant number of positive results were obtained. However, the percent positive in individuals born in the decades from 1921 to 1940 (89.2%) is somewhat greater than that in individuals born in 1941 to 1970 (65.2%). Both individuals born prior to the 1920s were vaccinated more than once, as was the one born between 1971 and 1980.

Of the 60 sera (44 were EIA positive and 16 were EIA negative) tested by SN, 59 (98.3%) were negative. The one SN-positive serum was from an individual born in 1948 and was negative by EIA.

Fifty-six sera from individuals under 30 years of age and presumably never vaccinated were also tested by EIA (control group). To our knowledge, none of the individuals were in the Armed Forces. In addition, they did not remember a previous smallpox vaccination. Of these 56 samples, 52 (92.9%) were negative and 4 (7.1%) were positive by EIA.

**DISCUSSION**

Two hundred four sera were tested for vaccinia virus antibody. Of these, 165 (80.9%) sera were from individuals who had been vaccinated once and 39 (19.1%) were from individuals who had been vaccinated at least twice. One hundred forty-three (70.1%) sera retained some evidence of vaccinia virus antibody as determined by EIA. Sixty-one (29.9%) sera were negative, including those from 8 of 39 individuals vaccinated at least twice. Of the 143 EIA-positive sera, 112 were from individuals (78.3%) vaccinated only once and 31 (21.7%) were from individuals vaccinated at least twice.

While the intent of the study was to determine the number of individuals who still had antibody after a single vaccination, there were 39 sera from individuals vaccinated more than once. It was of interest, therefore, to ascertain if there was any difference in residual antibody between single and multiple vaccinations. Interestingly, 67.9% of the 165 individuals vaccinated once had antibody, while 79.5% (31 of 39) of those who received multiple vaccinations were antibody positive. This suggests that multiple vaccinations may provide longer-lasting immunity against smallpox infection.

Inasmuch as the intent of the study was to determine the presence or absence of vaccinia virus antibody, the sera

<table>
<thead>
<tr>
<th>Decade of Birth</th>
<th>No. of sera (%)</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1920 or earlier</td>
<td>2 (1.0)</td>
<td>0</td>
</tr>
<tr>
<td>1921–1930</td>
<td>15 (7.4)</td>
<td>2</td>
</tr>
<tr>
<td>1931–1940</td>
<td>18 (8.8)</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>1941–1950</td>
<td>31 (15.2)</td>
<td>16 (7.8)</td>
</tr>
<tr>
<td>1951–1960</td>
<td>52 (25.5)</td>
<td>24 (11.8)</td>
</tr>
<tr>
<td>1961–1970</td>
<td>24 (11.8)</td>
<td>17 (8.3)</td>
</tr>
<tr>
<td>1971–1980</td>
<td>1 (0.5)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>143 (70.1)</td>
<td>61 (29.9)</td>
</tr>
</tbody>
</table>

* All 204 positive and negative sera, regardless of the number of times the individual had been vaccinated, are included. Of the 143 (70.1%) individuals that were EIA positive, 112 had received only one vaccination and 31 had been vaccinated more than once.

* Values in parentheses are percentages of the total number of samples (204).
were screened at a single dilution without determining end-point titers.

The data presented for the number of positives per decade of age show that the greatest percentage of EIA positives was among the number of donors who were born in 1921 to 1970. Considering that the number of sera collected from individuals with earlier or later birth dates was not significantly different, it is not clear why there are more positives among individuals born between 1921 and 1940 than among those born in 1941 to 1970. This may be a reflection of the number of sera tested in each decade or perhaps chance or coincidence.

The issue of smallpox protection is moot and would require additional study. If antibody detectable by EIA is protective, these results would suggest that individuals with some humoral antibody 30 to 35 years after vaccination might be protected against a bioterrorism attack with smallpox virus. It was of interest, therefore, to compare vaccinia virus antibody detection by EIA with detection by another serological test, the SN test. Of the 60 sera tested for SN antibody, the only SN-positive sample was a single EIA-negative serum. The failure to demonstrate SN antibody in the EIA-positive sera brings into question the protective capability of antibody detected by EIA. One may also ask why the one serum negative by EIA is SN positive. Additional testing with other immunological methods would be required to determine what constitutes protection.

It is highly probable, inasmuch as the members of the genus Orthopoxvirus are all closely related antigenically, that if there is protection against vaccinia virus, there is protection against the other members of the genus Orthopoxvirus (7, 8). Jezek et al. (7) indicate that smallpox vaccination protects against monkeypox. Smallpox virus is only one of the poxviruses considered in terms of bioterrorism: poxviruses other than variola and vaccinia (cowpox) viruses, including camelpox virus, monkeypox virus, lówlpox virus, and others, are recognized by the biological community (8).

Perhaps a smallpox attack would precipitate a protective anamnestic response or reveal a reduction in disease severity in EIA-positive individuals. If this did occur, then any smallpox outbreak in the population would be predominantly in the younger (nonvaccinated) age groups. The presence of antibody, as detected by EIA, in a large segment of the population may indicate that there is more protection to smallpox than originally thought.

The EIA control group of 56 individuals with no known history of vaccination indicated that positives do occur. Of the 56 sera, 52 (92.9%) were EIA negative. The four (7.1%) EIA-positive individuals may have been unknowingly vaccinated. As mentioned above, many of the tested subjects stated they were vaccinated, but the exact dates were vague. It may be that there were a number of individuals who have been vaccinated but are unaware of the event.

The results presented here indicate the need to explore what immune response, by EIA, SN, or other tests, is a true indication of protection from infection by the smallpox virus. The presence of a cell-mediated immune response to vaccinia virus in individuals vaccinated before 1980 should be studied in order to evaluate the results reported here. Cell-mediated immunity may reflect the true persistence of immunity to smallpox infection. In retrospect, the identification of vaccination scars at the time of phlebotomy might have been useful.

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REFERENCES