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Original article

Activity of vaccinia virus-neutralizing antibody in the sera of smallpox vaccinees

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Abstract

Individuals vaccinated against smallpox maintain substantial antiviral antibody responses for many years after vaccination. In this study, we examined the ability of antiviral antibodies from 104 unique serum samples to neutralize the two infectious forms of vaccinia virus, intracellular mature virus (IMV) and extracellular enveloped virus (EEV). While we found direct correlations between antiviral antibody titers and the ability to neutralize IMV and EEV, correlation with EEV neutralization was weaker. To determine factors that may influence more varied EEV neutralization within a vaccinated population, we asked the following questions. (1) Does vaccinia virus-neutralizing ability remain constant over time? (2) Do multiple vaccinations boost IMV and EEV neutralization activity? We found that serum from vaccinated individuals retained ability to neutralize EEV for a relatively long time, but there was a significant drop in EEV neutralization ability in the third decade after vaccination. While all vaccinees maintained some ability to neutralize IMV, a number of individuals lost the capacity to neutralize EEV. Interestingly, the ability to neutralize either virus form was not altered by the number of vaccinations received. Since it is likely that neutralizing antibodies against both IMV and EEV are required for maximal protective immunity, a loss of anti-EEV-neutralizing ability may warrant the revaccination of individuals who have been vaccinated > 20 years ago, should widespread pre-event smallpox vaccination be instituted.

Keywords: Smallpox; Human; Antibody

1. Introduction

The potential use of variola virus as a weapon of bioterrorism has raised questions about the level of protection conferred by remote smallpox vaccination. Since routine vaccination ended in the 1970s, the majority of the previously vaccinated population (~90% of Americans over the age of 35; 2000 United States Census Bureau) has not been exposed to vaccinia virus for at least 25–30 years. Recently, Hammarlund et al. [1] provided compelling evidence that while T-cell responses gradually diminish, antibody levels remain constant up to 75 years after smallpox vaccination. Several additional studies conducted prior to the eradication of smallpox illustrate a positive correlation between antibody titer and resistance to infection [2–4]. Indeed, the detection of vaccinia virus-specific antibodies has been suggested as a marker of adequate protective immunity [5]. However, there are two infectious forms of vaccinia virus, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). EEV is felt to be responsible for dissemination of vaccinia in vivo [6]. IMV represents the vast majority of progeny virus formed during an infection, and anti-vaccinia virus antibody and neutralization studies are mainly a measure of activity against IMV. For example, Hammarlund et al. [1] found a direct linear relationship between virus-specific antibodies quantitated by ELISA and the ability of those antibodies to neutralize IMV. Nevertheless, it has been shown that while inactivated virus produces high levels of neutralizing anti-

Abbreviations: EEV, extracellular enveloped virus; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; IMV, intracellular mature virus; NT_{30}, reciprocal of the serum dilution required for half-maximal EEV neutralization; NT_{50}, reciprocal of the serum dilution at which half the IMV plaques were neutralized; pfu, plaque-forming units.

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body against IMV, it fails to induce protective immunity [7,8]. Thus, in this study, using the well-characterized panel of serum used in the Hammarlund study, we examined whether virus antibody titers and IMV neutralization correlate with EEV-neutralizing capacity. We address two specific questions: (1) does virus neutralization remain stable over time? and (2) do multiple vaccinations affect the neutralization capacity of anti-vaccinia antibodies?

2. Materials and methods

2.1. Human serum

The demographics of the study population were previously described [1]. Additional serum was obtained from 22 volunteers vaccinated 2 months previously. Specimens were obtained from individuals at various years after vaccination (with number of volunteers in parentheses): 2 months ($n = 24$), 1 year ($n = 7$), 5–19 years ($n = 8$), 20–29 years ($n = 15$), 30–39 years ($n = 26$), ≥ 40 years ($n = 23$). The numbers of vaccinations per individual (with number of volunteers in parentheses) were as follows: 0 ($n = 6$), 1 ($n = 49$), 2 ($n = 33$), 3 ($n = 13$), 4 + ($n = 9$). All the samples used in this study were obtained from volunteers who had provided written informed consent under approval by the Institutional Review Board of Oregon Health Sciences University, and use of these human samples was approved by the University of Pennsylvania IRB.

2.2. Viruses

IMV was sucrose gradient purified following standard techniques [9]. Freshly prepared EEV was obtained from serum-free media (Opti-MEM) of RK-13 cells infected for 48 h with vaccinia virus strain IHDJ. The harvested media containing EEV was stored at 4 °C and used within 1 week after the primary infection. EEV titers in the media in the presence of anti-IMV neutralizing monoclonal antibody routinely approximated 10^6 plaque-forming units (pfu)/ml.

2.3. IMV and EEV neutralization assays

Six fourfold dilutions (beginning at 1:4) of heat-inactivated human serum were mixed with IMV (~200 pfu per sample), and six twofold sera dilutions (beginning at 1:4) were mixed with EEV (~200 pfu per sample). EEV was added along with the anti-IMV neutralizing antibody, 2D5, at a 1:1000 dilution [10,11]. Samples were incubated for 1–2 h at 37 °C and then added to confluent monolayers of BSC-1 cells in 6-well plates and incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. The inoculum was removed, the wells overlaid with media containing 2.5% heat-inactivated fetal calf serum and 1% carboxymethylcellulose, and the plates incubated for 48 h. The overlay was removed, the cells stained with 0.1% crystal violet, and the plaques counted. All serum dilutions and plaque reduction assays were done in duplicate. The reciprocal of the serum dilution at which half of the IMV plaques were neutralized (NT_{50}) was determined for each specimen [1]. For EEV neutralization, we chose to analyze the NT_{30} of EEV because of the difficulty in neutralizing this form of vaccinia virus [12–14]. We selected this value because at the highest concentration of serum we tested (1:4), we found the mean percent neutralization of EEV by all vaccinated individuals to be approximately 60%. Thus, the NT_{30} represents the reciprocal of the serum dilution required for half-maximal EEV neutralization.

2.4. Statistical analysis

Kruskal–Wallis test was performed and identified that statistically significant differences existed between groups within each data set. Then pair-wise comparison between each group in a data set was done by a nonparametric Wilcoxon test. Correlations and 95% confidence intervals were examined by Spearman rank correlation coefficient.

3. Results

3.1. Correlations between vaccinia virus-specific antibody production and virus neutralization

Hammarlund et al. [1] previously quantitated the virus-specific antibody levels of these individuals by enzyme-linked immunosorbent assay (ELISA). One hundred ELISA Units (EU) was considered the lowest positive titer and was shown to have 100% specificity and sensitivity (0 of 26 unvaccinated controls scored ≤ 100 EU; 98 of 98 vaccinated samples scored ≥ 100 EU). A direct linear correlation was made between the virus-specific antibody responses and IMV-neutralizing titers (Log NT_{50}) of the vaccinated population [1]. We repeated this using additional serum samples and similarly found a strong correlation between the virus-specific antibody responses and IMV-neutralizing titers (Log NT_{50}) of the vaccinated population ($R^2 = 0.638$ (0.505, 0.740 (95% confidence interval)); $P < 0.0001$) (Fig. 1A). Next, we examined the relationship between virus-specific antibody levels and EEV neutralization. While EEV-neutralizing titers were also found to correlate with virus-specific antibody levels ($R^2 = 0.573$; (0.424, 0.689 (95% confidence interval)); $P < 0.0001$), the correlation was weaker, and 13 of 104 vaccinees (12%) had positive virus-specific antibody levels and yet had an un-measurable NT_{30} (Fig. 1B). Of note, a further eight vaccinees (7.5%) had Log NT_{30} values of ≤ 0.5, which corresponds to < 15% EEV neutralization at the highest serum dilution tested. We next examined the correlation between EEV neutralization and IMV neutralization. While here too, there was a correlation between EEV neutralization and IMV neutralization ($R^2 = 0.425$; (0.257, 0.566 (95% confidence interval)); $P < 0.0001$), 12% of individuals with high Log NT_{50} values had an un-measurable EEV Log NT_{30} (Fig. 1C).