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The smallpox vaccine induces an early neutralizing IgM response

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ABSTRACT

The antibody response elicited after immunization with vaccinia virus (VacV) is known to be sufficient to confer host protection against VacV or smallpox. In humans it has been shown that such anti-VacV antibody production can be sustained for decades. Nevertheless, little is known about the kinetics and the role in protection of the early antibody response after vaccination. In this study we identify VacV neutralizing IgM antibodies as early as 4 days after infection of C57BL/6 mice. Most of this IgM production is T cell dependent and predominantly independent of the germinal center reaction (SAP/SH2D1A independent). Importantly, the IgM neutralized both infectious forms of VacV: the intracellular mature virion (MV, IMV) and the extracellular enveloped virion (EV, EEV). Moreover, in mice primed with MHCII restricted peptides, an increase in the total VacV neutralizing antibody titers was seen, a large component of which was neutralizing IgM against the same protein from which the priming peptide was derived. To further demonstrate the biological relevance of this early neutralizing response, we examined anti-VacV antibodies in humans after vaccination. Human subjects could be divided into two groups early after immunization: IgG^{hi} and IgG^{lo}. VacV IgM neutralizing antibodies were detected in the IgG^{lo} group. Taken together these results indicate that both in a small animal model and in humans an early neutralizing IgM response after VacV immunization is present and likely contributes to control of the infection prior to the development of a robust IgG response.

1. Introduction

Immunization with the smallpox vaccine results in long term protection against VacV itself and other orthopoxviruses, most importantly variola (smallpox). The protective activity is mainly due to anti-VacV neutralizing antibodies [1], which are continuously produced for decades after vaccination [2–5]. Many studies have described roles of different innate and adaptive immune response cell types in the control of a primary VacV infection or vaccination [1,6,7]. However, while B cells and antibodies are crucial for the long term protection, the roles of B cells and antibodies in the primary response to the smallpox vaccine remain less clear [1,8].

Mice deficient of B cells (μMT/μMT) have an increased susceptibility to VacV infection, and higher viral titers are found in μMT mice compared to wild-type (WT) controls [6], suggesting that the early antibody production has an important role in the control of the infection. Furthermore, antibodies are essential for control and clearance of the related poxvirus, ectromelia (mousepox) [9–11], while T cell responses are also critical for control of a primary ectromelia infection [9,12]. VacV has two different infectious forms, the intracellular mature virion (IMV, MV) thought to be primarily responsible from host to host infection, and the extracellular enveloped virion (EEV, EV), responsible for much of the viral dissemination within the host [13]. Studies have demonstrated that antibodies directed against either virion form are protective in mice [14–17], and we have recently demonstrated that B5-specific anti-EV antibodies can efficiently clear virally infected cells, illustrating that the protective value of anti-VacV antibodies is not limited to free virions [18]. Moreover, in the rhesus macaque model it has been shown that B cells but not CD4+ nor CD8+ T cells are essential for protection against a lethal challenge with monkeypox virus [19]. All of these reports highlight the importance of antibodies in the control of VacV and related poxviruses.

While it is clear that neutralizing antibodies are a very potent mechanism for protection against VacV, monkeypox, and variola, the neutralizing IgG response is relatively slow to develop after a primary infection or immunization. In vaccinated humans, neutralizing IgG is reported at 2–3 weeks after vaccination [1,20,21], after the resolution of the primary infection and lesion. In mice, significant anti-VacV IgG titers have not been reported earlier than 8 days post-infection (i.p.), which is after the bulk of the infection has been cleared. Therefore, it appears that the neutralizing IgG response develops too late in humans and mice to be of significant value in controlling a primary VacV infection/immunization. Given that, is there any role for neutralizing antibodies in control of the primary VacV infection?

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The study reported here demonstrates for the first time that there is an early induction of neutralizing IgM after VacV immunization of mice and humans, and implicates a protective role for IgM during the primary response.

2. Materials and methods

2.1. Mouse procedures

C57BL/6J (B6 or WT) and C57BL/6J Ia^b-$Ea^{-/-}$ (MHC class $II^{-/-}$) mice were purchased from The Jackson Laboratory. $SAP^{-/-}$ mice [22] back-crossed into C57BL/6J [23] were bred in-house.

Mice were infected with the vaccinia virus Western Reserve strain (VacV) by bilateral intraperitoneal (i.p.) injection of 2×10^6 PFU total with standard purified VacV stocks. For peptide immunizations, 30 μg of peptide was emulsified in complete Freund adjuvant (CFA) and injected subcutaneously between the scapula, and 11–13 days after peptide immunization were infected with VacV. Serum was obtained by retro-orbital bleed at determined time points post-infection. To determine the kinetics of the appearance of neutralizing antibodies, two groups of WT mice were infected with VacV, one group was bled on odd days and the other was bled on even days. All mice were maintained in an accredited facility at LIAI, and all the experiments were conducted in accordance with approved animal protocols.

2.2. Human study subjects

A cohort of 14 normal healthy volunteer donors were immunized for the first time with the smallpox vaccine (Dryvax) and blood samples were obtained at the indicated time post-vaccination. The gender distribution of the cohort was ~50:50, within an age range from 23 to 60 years.

2.3. Plasma and serum

Plasma samples from the human subjects and sera from VacV infected mice was stored as aliquots at −80 °C. To eliminate IgM antibodies, aliquots were treated with an equal volume of 0.1 M 2-mercaptoethanol (2-ME) in PBS for 1 h at room temperature, as described [24–26].

2.4. MV production

VacV MV stocks were produced as described in [27]. Briefly, MV were in grown on HeLa cells in D-10 (Dulbecco's modified Eagle medium [DMEM] plus 10% heat inactivated fetal calf serum [FCS] plus penicillin/streptomycin/glutamine) in T175 flasks (Falcon; Becton Dickinson), infecting at a multiplicity of infection (MOI) of 0.1–0.5. Cells were harvested at 2.5–3 days, and virus was isolated by rapidly freeze-thawing the cell pellet three times in a volume of 2.3 ml DMEM or RPMI supplemented with 1% heat inactivated FCS. Cell debris was removed by centrifugation ($700 \times g$, 8 min). Clarified supernatant was frozen at −80 °C as virus stock. Titers of VacV stocks were determined with VeroE6 cells (~2×10^8 PFU/ml). Purified VacV stocks were made by sonication of the VacV stock (40 s) using a water sonicator (Branson Ultrasonics, CT) and layered over 36% sucrose in TM buffer (10 mM Tris–HCl [pH 7.4], 5 mM $MgCl_2$). VacV was centrifuged (SW28 rotor) at 13,500 rpm ($33,000 \times g$) for 80 min at 4 °C. The VacV pellet was resuspended in 1 ml TM buffer and then brought up to 10 ml with DMEM medium supplemented with 1% of heat inactivated FCS. Purified VacV was stored at −80 °C.

2.5. EV production

VacV EV was prepared as described elsewhere [18]. Briefly, HeLa cells were cultured in D-10 in T75 flasks (Falcon, Becton Dickinson) at 90% confluence and infected with VacV at a MOI of 0.5. The medium containing EV was harvested at 2 days, and virus was isolated by centrifugating twice ($450 \times g$, 8 min) to remove cells and debris. Clarified supernatant was used immediately or stored at 4 °C for a maximum of 3 weeks. EV VacV stocks were titrated on VeroE6 cells (~5×10^5 PFU/ml).

2.6. MV neutralization assay

Titration of VacV MV neutralizing antibodies, was performed according to Newman et al. [28]. Briefly, VeroE6 cells were seeded at 2×10^5 cells/well into 24-well Costar plates (Corning, Inc., Corning, NY) and used the following day (75 to 90% confluence). Total or IgM depleted plasma or serum was incubated overnight with 50 μl of freshly sonicated VacV (10^4 PFU/ml) at 37 °C with 5% CO_2. Plasma from non-vaccinated human individuals or sera from naive mice were treated under the same conditions and used as negative controls. Multiple wells of VacV-alone controls were always used. Medium was aspirated, and the samples were added and allowed to adsorb for 60 min at 37 °C. Then the cells were rinsed with warm phosphate-buffered saline (PBS) and 1 ml of D-10 medium was added. Plates were incubated for 40–48 h and then fixed and stained with 0.1% crystal violet in 25% reagent alcohol (90% ethanol, 5% methanol, 5% isopropanol) to count viral plaques.

2.7. EV neutralization assay

VacV EV neutralization assay was described elsewhere [18]. Briefly, all the samples and FCS were heat inactivated (56 °C, 30–60 min) prior to use to eliminate complement function. VeroE6 cells were prepared in 24-well Costar plates as described. The samples were incubated for 30 min at 37 °C with an equal volume (50 μl) of EV stock (1:100–1:400 dilution) supplemented with 10% (final concentration) sterile baby rabbit complement (Cedarlane Laboratories, Ontario, Canada) and rabbit anti-L1 (1:25–1:100 final). Anti-L1 was used to neutralize the MV present in the EV stock [18,29]. EV supplemented with anti-L1 antibody alone was regularly used in each assay +/− baby rabbit complement as negative controls. Medium from 24-well plate wells was aspirated and samples were added and allowed to adsorb for 45 min at 37 °C. After this step the plates were treated as in the MV neutralization assay.

In both neutralization assays the $PRNT_{50}$ was defined as the reciprocal of the last dilution of the plasma that reduced the average number of plaques by 50% compared to the mean number of VacV-alone plaques.

2.8. VacV proteome microarray

Production and use of protein microarrays is described elsewhere [17,27,30]. Briefly, VacV proteome arrays were probed with the sera or plasma samples and bound antibodies were detected with a Cy3-conjugated goat anti-mouse IgG (heavy and light chains) secondary antibody (Jackson ImmunoResearch). The arrays were examined in a GSI Lumonics ScanArray 4000 confocal glass slide scanner and intensities were quantified using QuantArray software. Results were quantified as relative fluorescent units (RU) over background. Signals from three negative control spots were averaged, and that background signal was subtracted from all spots on the array to give RU.