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Measurement of antibody responses to Modified Vaccinia virus Ankara (MVA) and Dryvax® using proteome microarrays and development of recombinant protein ELISAs

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Introduction

The eradication of smallpox using the related orthopoxvirus, vaccinia virus, remains one of the medical triumphs of the 20th century. Although vaccinia was an effective vaccine, it is associated with the risk of rare but serious adverse events that may occur more frequently in immunodepressed individuals, such as the very young or elderly, women who are pregnant, transplant recipients undergoing therapeutic immunosuppression, and patients who have HIV/AIDS [1]. Until recently, Dryvax® (Wyeth), a lyophilized calf lymph virus derived from the New York City Board of Health (NYCBH) strain, was the only licensed smallpox vaccine in the US. Dryvax is clonally heterogeneous, and was produced on the skin of calves and thus carried the risk of contamination with other pathogens. These production methods are now considered unacceptable. A recently produced clonal derivative of Dryvax produced in serum-free tissue culture, ACAM2000 (Acambis), was determined to be non-inferior to Dryvax based on take rate. Millions of doses were purchased for the Strategic National Stockpile for individuals at high risk of exposure in the event of a bioterrorist attack [2–4], and in 2009 Dryvax was withdrawn.

Although an effective replacement, ACAM2000 poses the same safety concerns as Dryvax. Modified vaccinia Ankara (MVA) is a severely attenuated vaccinia virus that may be more suitable for individuals where use of replication competent vaccines is contraindicated. MVA was derived in the 1960s from the Turkish vaccine strain, Chorioallantoid Vaccinia Ankara (CVA), by over 570 passages in chicken embryo fibroblasts [5, 6]. As a result, MVA is unable to replicate in most mammalian cells [7]. Sequencing revealed loss or truncation of 31 open reading frames in six major deletions, chiefly among immunomodulatory genes located in the terminal regions of the genome [8–11]. In mammalian cells the infectious cycle is aborted at the late stage of infection resulting in the absence of cytopathic effect or plaque formation [6]. This results in a highly attenuated phenotype in humans and other

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mammals that allows MVA to retain immunogenicity [12–17] but with a significantly reduced incidence of adverse events [18–20]. MVA was used in Germany in over 120,000 individuals during the eradication campaign without any adverse events. MVA also expresses heterologous genes at high levels [21, 22]. Because of the excellent safety profile and its large, non-integrating genome makes MVA an attractive option for the delivery of vaccine antigens for the prevention and treatment of other human ailments, such as HIV/AIDS [23, 24], influenza [25], malaria [26], tuberculosis [27, 28] and various cancers [29, 30].

Recent studies have shown that an antibody response against vaccinia virus is crucial for induced protection [31, 32]. Monitoring the immune response elicited by smallpox vaccines is conventionally based on the measurement of antibodies by ELISA and the plaque reduction neutralization test (PRNT). Our laboratory has developed a proteome microarray platform that has been useful for identifying antibody targets in vaccinia [33–35] and a variety of other pathogens [36–44] for the development of subunit vaccines and improved serodiagnostic tests. Target antigens identified by the array approach can be then purified in large quantities for preclinical protection studies, prototype immunoassay fabrication and product development. Recombinant protein based immunoassays offer certain advantages over whole organism based tests. In particular, they may allow development of tests that can discriminate more precisely between different stages of infection, strains of pathogen, or disease states. Such tests may be based on single antigens or multiplexed combinations.

Over the past few years, the NIAID/NIH has sponsored several multi-center clinical trials looking at the safety and immunogenicity of Dryvax and MVA. From these trials, >2,000 sera from several different protocols have been probed against the proteome arrays. In this report we focus on dominant antigens detected in the human profiles with a view to developing immunoassays for MVA and Dryvax, particularly multiplex ELISAs, which could assist with vaccine development. Antigens were selected for assay development based on dominance in the antibody profile and manufacturing considerations in *E. coli*. We report on six as lead candidates, comprising three membrane and three non-membrane antigens from the intracellular mature virion [45–47], which we evaluated in ELISAs with sera from MVA and DryVax vaccinees. We also aimed to determine whether, and how, previous smallpox vaccination influences a subsequent MVA response.

Materials and Methods

Human sera and protocols

Sera from multiple NIAID/NIH-sponsored clinical trials of MVA and Dryvax were obtained for testing. All clinical trials were conducted with appropriate consent and approval of local IRB and ethics committees. Sera were supplied frozen on dry ice and coded as to the vaccination regimen. No patient identifiers were provided. Once thawed, sera were aliquoted; one aliquot was mixed 1:1 with glycerol and stored at -20°C and used for probing, and the remaining aliquots maintained at -80°C . Vaccinia immune globulin (VIG) was a gift from Cangene Inc.

Protein microarrays

The strategy used in this study was to use antigens of the vaccinia virus Western Reserve strain (VACV-WR) to detect cross-reactive antibodies to vaccine strains MVA and Dryvax in human vaccinees. Microarrays for this study were constructed essentially as described previously [33, 34, 48], with the exception that the VACV-WR plasmid collection used for protein expression in this study was generated from scratch. This new collection has been characterized and used for recent T cell studies [49, 50] although this represents the first

serological study to use this collection. Briefly, vaccinia virus WR genomic DNA was used as a template for PCRs to amplify individual open reading frames (ORFs), which were then cloned into a T7 expression vector by use of homologous recombination. All proteins were expressed from purified plasmids in *Escherichia coli*-based coupled in vitro transcription/translation reaction (IVTT; RTS 100 kits from Roche). Reactions representing 210 different VACV-WR proteins were printed without further purification as 16×16 arrays onto 16-pad nitrocellulose-coated FAST slides (Whatman) using an Omni Grid 100 microarray printer (Gene Machines). All arrays are printed with serial dilutions of human IgG as a control for the secondary antibody. We also printed purified EBNA-1 (Epstein-Barr nuclear antigen 1), which is recognized by most human sera and provides a useful way to monitor serum quality and experimental procedure. Multiple negative controls spots comprising IVTT lacking template DNA, were also printed on each array. Arrays were printed over several print runs. Protein expression was verified using antibodies to N- and C-terminal epitope tags (polyhistidine and hemagglutinin epitope) as described [33]. To monitor consistency between runs we probed slides at the beginning, middle and end of each print run with aliquots of VIG.

For microarray probing, the arrays were first blocked for 1h in protein array blocking buffer (Whatman), during which time human sera were diluted 1/100 in blocking buffer containing *E. coli* lysate (Antigen Discovery Inc, Irvine CA) to a final concentration of 10mg/ml protein to block anti-*E. coli* antibodies, and incubated for 30 minutes with constant mixing prior to applying to the arrays in triplicate. Arrays were probed overnight at 4°C with gentle agitation. The following day, slides were washed 4× in Tris-buffered saline containing 0.05% Tween-20 (T-TBS). Biotinylated anti-human IgG (Jackson) was used as secondary antibody, followed by streptavidin-PBXL3 conjugate (Martek Biosciences), both used at 1/200 in blocking buffer. After being washed 3× in T-TBS and 3× in TBS, slides were dipped in distilled water and air-dried by brief centrifugation. Slides were scanned using a Perkin Elmer ScanArray Express HT microarray scanner (PerkinElmer) and fluorescence intensities quantified using ProScanArray Express software (PerkinElmer). All signal intensities are corrected for spot-specific background.

Protein purification

For antigens selected for scale-up purification, plasmids were transformed into BL21 cells, colony selected and sequence verified. Master cell bank glycerol stocks were made and used to initiate starter cultures grown overnight in LB broth. Antigens were expressed as inclusion bodies by growing the bacteria overnight at 30°C/300rpm in 100ml auto-induction medium (MagicMedia from Invitrogen) [51], supplemented with 100µg/ml kanamycin. The following day, were cells harvested by centrifugation and lysed in BugBuster™ supplemented with 1µl/ml benzonase and 1,000 U/ml lysozyme (all from Novagen), and one protease inhibitor pill per 50ml (Complete from Roche). After washing the inclusion bodies three times in lysis buffer (once in 1× and twice in 0.1×), the inclusion bodies were gently solubilized by overnight incubation in 0.1% SDS in PBS. Proteins that were not solubilized (clear) at this time were solubilized by raising the SDS concentration to 0.2–0.4%. Free SDS was then precipitated on ice by adding 40mM KCl (final concentration) and pelleted by centrifugation. Finally, supernatant containing solubilized proteins was passed through a 0.2µm filter and glycerol added to 10% prior to storage of aliquots at –80°C. Some proteins were also purified by conventional nickel-chelate affinity purification using commercially available columns (Novagen His-Bind). Protein expression was monitored by SDS PAGE and Western blot with monoclonal anti-histidine and anti-hemagglutinin antibodies as described. Proteins were quantified using the Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

Antigens were coated onto microtiter plates (Thermo Scientific) at 3–0.3µg/ml (50µl/well) in TBS overnight at 4°C. The following day the plates were washed four times in TBS-Tween 20 (T-TBS, USB) and blocked with 300µl/well casein/TBS blocking buffer (Thermo Scientific) for 1–2h. Blocking buffer was then decanted and the plates air dried and stored in desiccated foil pouches at 4°C until required for use. For ELISA assay, sera were diluted to 1/200 in blocking buffer supplemented with *E. coli* lysate (Antigen Discovery Inc) to a final concentration of 10% and incubated for 30 min before adding to the plate. Plates were incubated for 1h with gentle rocking at room temperature (RT), then washed in T-TBS again, and goat anti-human IgG-HRP conjugate (Bethyl) diluted 1/25,000 in blocking buffer was added to the wells and incubated for 1h at RT. After washing in T-TBS, plates were developed by adding 100µl/well SureBlue Reserve™ TMB peroxidase substrate (KPL) for 10min in the dark. Development was stopped by the addition of H₂SO₄ and OD read at 450nm in a Multiskan FC plate reader.

Data handling

Statistical analyses were performed using the R (<http://www.r-project.org>) and SAS (<http://www.sas.com/>) statistical software. In order to stabilize the variance observed in microarray signals [52, 53], the vsn normalization method implemented as part of the Bioconductor suite (www.bioconductor.org) was applied to the quantified array intensities. This procedure removes heteroskedacity [54–56] and corrects non-specific noise effects such that control probe variance is minimized. This calibration has been shown to be effective on a number of platforms [57]. Comparisons between groups were performed using a Bayes regularized t-test adapted from Cyber-T for protein arrays [54, 58], which has been shown to be more effective than other differential expression techniques [59]. To account for multiple comparison conditions, the Benjamini and Hochberg (BH) method was used to control the false discovery rate [60]. After Benjamini and Hochberg correction, p-values <0.05 were considered significant, and the corresponding protein is considered differentially reactive. Receiver Operator Characteristic (ROC) analyses were performed with ELISA data for each antigen by testing the sum OD-450nm for each subject as threshold cut off to discriminate pre and post vaccination. Sensitivity and specificity values were calculated for each cut-off and the data plotted to give an ROC curve.

Results

Dominant antigens of Dryvax and MVA profiles are partially overlapping

In the study reported here, we focused on dominant antigens in the human MVA and Dryvax profile to provide a priority list of antigens for scale-up purification and immunoassay development, and to compare with earlier published work on MVA with smaller sample numbers [34, 35]. We also aimed to determine whether and how previous smallpox vaccination influences a subsequent MVA response. An overview of the protocols sampled is listed in Supplemental Data, Table 1. Studies that have been published [61–65] are indicated in the table. In each case sera were obtained on the day of vaccination (d0) and at least one other time point, usually d42–56 for the peak MVA response and d28 for the peak Dryvax response. When probing arrays, it was common to observe antibody binding to some antigens on d0, even though the donors were naïve and had none of the exclusion criteria that would indicate previous vaccination. Thus, when analyzing vaccinia naïve individuals in this study, we routinely subtracted d0 signals from all time points.

We first used proteome microarrays to identify the antigens that gave the strongest antibody responses to MVA and to Dryvax. Shown in Fig. 1A and B are representative data from a single clinical trial (protocol #1). Vaccinia-naïve volunteers were given two MVA

inoculations delivered subcutaneously on d0 and d28 (Fig. 1A) or given a single dose of Dryvax administered percutaneously on d0 by scarification (Fig. 1B). After the first MVA inoculation, the IgG response peaks around d28. Signals to the virion assembly protein WR118/D13L are consistently highest among the MVA vaccinees, with responses to IMV membrane proteins WR113/D8 and WR101/H3L, and EEV membrane proteins WR187/B5R and WR157/A34R also prominent. After the second inoculation on d28 (panel A), there is a dramatic increase in antibody titers, which peak around d42 and decline thereafter. The profile is again dominated by antibodies to WR118/D13L, WR113/D8L, WR101/H3L, WR187/B5R and WR157/A34R. The Dryvax response (panel B) first appears on d8-d14. Signals to A-type inclusion protein, WR148, WR118/D13L, core protein WR070/I1L, IMV membrane proteins WR101/H3L, WR150/A27L and EEV membrane hemagglutinin WR181/A56R, are consistently the strongest among vaccinees. The signals to these different antigens arise simultaneously, and several are still rising at the last sampled time point (d28). The most noticeable difference between MVA and Dryvax profiles is the response to WR148, which is immunodominant in the Dryvax profile but absent from the MVA profile. The cowpox ortholog of WR148 encodes a 150kDa protein that forms protein-dense A-type inclusions (ATIs) in infected cells. In vaccinia, the WR148 gene is truncated to 94 kDa [66]. Other related ATI ORFs in vaccinia, WR145, 146, and 147, lack promoters and are thought not to be expressed in vaccinia, although we see sporadic reactivity to WR147 and WR146 in Dryvax vaccinees. The ATI region is deleted from MVA [8]. The differences between the MVA and Dryvax profiles are discussed further below.

Shown in Fig.1C is a Dryvax profile from an independent separate clinical study (protocol #2 [61]) to illustrate the consistency of profiles from different clinical trials. The top antigens are again ranked independently, and by visual inspection results in a very similar profile to that shown in Fig 1B. When the peak antibody profiles (d28) were compared by T-test, none of the antigenic responses were significantly different between the two populations (data not shown).

Discriminatory and non-discriminatory antigens are revealed by T tests

One aim of this study was to develop ELISAs using both strain specific and shared antigens to monitor MVA and Dryvax vaccination. There is considerable overlap between antigens in the peak MVA and Dryvax profiles, which represent ideal antigens for monitoring a response to both vaccines. Responses that are consistently different between the profiles could be used to develop strain specific assays. We addressed this using T tests to identify significant and non-significant differences between the peak MVA and Dryvax profiles (d42 and d28, respectively). Shown in Fig. 2 are differentially ($p_{BH} < 0.05$) and non-differentially ($p_{BH} \geq 0.05$) recognized antigens in protocol #1 (same sera as Figs. 1A and B). The most discriminatory antigen (i.e., with the lowest Benjamini-Hochberg corrected p-value) was WR148. There is no corresponding ortholog in MVA and it is therefore considered discriminatory (serodiagnostic) for Dryvax. Other significant differences include WR118/D13L, WR070/I1L, WR181/A56R and WR150/A27L. Unlike WR148, all of these are retained in MVA. It is likely these are reflective of lower antibody titers associated with MVA infection, although we are aware that lower signals could reflect differential binding of MVA and Dryvax antibodies for the same WR antigen. Since Dryvax is non-clonal, we compared the amino acid sequence of ACAM2000 [2, 67], with the Acambis MVA, ACAM3000 [68, 69] using the Poxvirus Bioinformatics Resource Center (<http://www.poxvirus.org>). The homologies concerned are very high (Supplemental Data, Table 2) suggesting antibodies should cross-react with WR equally well, and that the lower signals for several antigens after MVA infection are related to antibody titer and is consistent with an abortive replication cycle. Reciprocally, antigens that were equally reactive in MVA and Dryvax (i.e., not significantly different) include the IMV membrane

proteins WR101/H3L and WR113/D8L, and the EEV membrane proteins WR187/B5R and WR052/F13L (Fig. 2). While there were several antigens potentially diagnostic of Dryvax, no single antigens were diagnostic of MVA. Two or more antigens would need to be used for MVA diagnosis; for example, a ratio H3L/A27 close to 1 would be diagnostic of Dryvax, whereas a ratio >100 would be diagnostic of MVA.

While the numbers of individual donors in each clinical trial are relatively small, larger sample sizes were obtained by pooling data from different trials. Data from two independent MVA, and three independent Dryvax arms, are compiled in Supplemental Data Fig. 1. The independent MVA arms were first confirmed to have no significant differences (not shown) and the data pooled. The Dryvax arms were similarly found to have no significant differences and pooled. A repeat T test analysis using data from the pooled MVA and Dryvax arms (n=89 vs. 68 donors, respectively) indicated the strongest discriminatory and non-discriminatory reactive antigens correspond closely to those found previously (Supplemental Data Fig. 2). In a few cases, reactive antigens that were not significantly different originally became significant in the larger data set, notably the EEV membrane protein WR187/B5R.

Frequency of recognition correlates with signal intensity/titer

We established above that, for a given protein on the array, changes in signal intensity over time or between different vaccines correlate with antibody titer. However, while antigens with the strongest signals represent lead candidates for immunoassay development, those with the greatest serodiagnostic utility are those that are recognized by as many individuals as possible (preferably all) after vaccination. It is known from previous proteome microarray studies that human antibody profiles in response to smallpox vaccination exhibit wide variation between individual donors, and that the frequency of recognition by a vaccine group varies between antigens [34, 35, 70]. We first determined recognition frequencies for different reactive antigens (both diagnostic and cross-reactive) in the pooled data set using a simple cut-off based on mean + 2SD of signals of the donors on d0 (Table 1). When ranked by frequency of recognition, this list reflects the antigens ranked by average signal intensity (Fig. 1), presumably as the strongest signals are unambiguous positives. The three most commonly recognized antigens in Dryvax vaccinees were WR118/D13L, WR101/H3L and WR113/D8L (97.1%, 92.6% and 91.2%, respectively). WR148, although seen by only 75% of Dryvax vaccinees when screened by arrays, remains the best discriminatory antigen. WR150/A27L is also a good discriminator based on frequency of recognition, which is consistent with the T tests (Fig 2). For MVA, the three most commonly recognized antigens were WR113/D8L, WR101/H3L and WR187/B5R (88.8%, 79.8% and 67.4%, respectively). Combining WR113/D8L with WR101/H3L and WR187/B5R progressively increased coverage from 88.8% to 95.6% and 98.5%, respectively. None of the antigens were recognized more commonly by MVA vaccinees compared to Dryvax.

Heterologous boosting in re-vaccinations

Routine smallpox vaccination using Dryvax was discontinued in the US in 1971. For individuals vaccinated prior to this, it is important to know what effect pre-existing immunity might have on the response to MVA. This has implications for the use of MVA both as a second-generation vaccine against smallpox, and as a recombinant vector for the delivery of heterologous antigens in immune populations. There are also wider implications for understanding immunological cross-reactivity between orthopoxviruses, as this is thought to be the mechanism by which protection against smallpox is engendered by vaccinia. In Fig 3A are shown antibody profiles before and after MVA vaccination in individuals known to have been vaccinated previously against smallpox. The signals on d0 are consistent with previous exposure to vaccinia, as evidenced by signals to WR148 and

other dominant antigens such as WR118/D13L, WR101/H3L and WR113/D8L. These antigens are candidates for development of immunoassays for measurement of pre-existing immunity (see below). After boosting with MVA, signals to WR118/D13L, WR101/H3L, WR113/D8L and other antigens increase (~3–6 fold). For antigens where there is high amino acid sequence identity (Supplemental Data, Table 2), MVA boosts preexisting immunity from previous vaccination against smallpox (“heterologous boosting”). In the case of WR148 no boosting occurs and the titers from the primary vaccination remain unchanged, whereas Dryvax revaccination (Fig 3B) engenders responses to several antigens *including* WR148 (~3 fold). For comparison, are shown the corresponding profiles for vaccinia-naïve individuals receiving MVA and Dryvax (Figs. 3C and D, respectively). The baseline (d0) signals are lower than the d0 signals in the previously vaccinated group as expected.

The effects of previous smallpox vaccination on the peak MVA profile can also be seen in Supplemental Data Fig. S1, where d42 profiles in naïve and previously vaccinated individuals are compared (panels B and C, respectively). While similar, the response to EEV membrane protein WR187/B5R does not appear to be boosted by MVA in previously vaccinated individuals. Reciprocally, the responses to WR070/I1L and WR150/A27L after MVA are stronger in the previously vaccinated group.

We would predict that pre-vaccination with MVA would also allow heterologous boosting by Dryvax. Such a study, shown in Fig. 4, was originally designed to determine whether MVA could be used to ameliorate the potential adverse effects of Dryvax while retaining immunogenicity. MVA was given on d0 and d28 and followed by Dryvax or placebo given on d112 (Fig. 4A and B, respectively). A control group received Dryvax alone (Fig 4C). For clarity, only 3 dominant antigens are shown: WR148 (deleted from MVA), and WR113/D8L and WR118/D13L as representative antigens shared by both vaccines. MVA profiles are essentially identical at the peak on d42 ($pBH < 0.05$; data not shown) and begin to decline thereafter. After a Dryvax boost on d112 (panel A) the signals to WR113/D8L and WR118/D13L (and other shared antigens that are not shown) are elevated, consistent with heterologous boosting. Without further boosting the signals continue to decline (Fig. 4B). Interestingly, it can be seen that a primary response to WR148 develops after a Dryvax boost of MVA-immune individuals (Fig 4A), although it was attenuated compared to primary Dryvax response in naïve individuals (Fig. 4C). Those that did respond to WR148 mounted a weaker response relative to the response to DVX in the absence of pre-existing immunity. Since pre-existing immunity to MVA lacks memory of the WR148 antigen, the Dryvax boost is analogous to a recombinant vaccinia where WR148 represents the ‘heterologous’ antigen. However, studies with true recombinants are needed to properly address concerns that preexisting immunity to vaccinia may interfere with the subsequent use of recombinant vaccinia for the delivery of heterologous antigens.

Production and evaluation of ELISAs using vaccine strain specific and shared antigens

On the basis of array data described above, six VACV-WR antigens were successfully purified and used to fabricate ELISAs: WR148, WR113/D8L, WR118/D13L, WR101/H3L, WR132/A13L and WR070/I1L. These comprise three intracellular mature virion (IMV) membrane proteins and three non-membrane proteins [45–47]. Each was chosen for their dominance in the MVA and/or Dryvax profiles and frequency of recognition by array. Expression levels in *E. coli* BL-21 cells, and ease with which pure protein could be obtained were also major considerations. Although not deliberately excluded, membrane proteins from EEVs were not among the proteins short-listed for further study due mainly to these manufacturing considerations. WR148, although expected to be reactive in Dryvax vaccinees only, was included as it is one of the most immunodominant antigens in Dryvax. The remaining 5 antigens were characteristic of both Dryvax and MVA profiles. The aims

were to determine how well the purified proteins performed for detecting responses to MVA and Dryvax vaccination, and the minimum number of antigens required to provide the best coverage. We also aimed to determine whether primary or secondary responses could be distinguished using one or more of the antigens.

WR148, WR118/D13L, WR132/A13L and WR070/I1L were expressed as inclusion bodies by overnight expression in *E. coli* BL21 cells. Following standard BugBuster® extraction and washing of the inclusion bodies, proteins were solubilized by overnight incubation in a low concentration (0.1 – 0.2%) of SDS. The remaining free SDS was removed by precipitation, and the proteins were sterile filtered (see Materials and Methods). WR101/H3L and WR113/D8L were purified from the soluble fraction of *E. coli* lysates using nickel-chelate affinity chromatography, as yields using the inclusion body approach were too low. Antigen coating concentrations were initially optimized using VIG, the IgG fraction of pooled hyperimmune sera. These coating concentrations were then verified against a panel of human sera from d0 and d28 after Dryvax inoculation. The optimal coating concentration for the majority of antigens was ~3µg/ml, although WR148 was more potent and coated at 0.3µg/ml (Supplemental Data, Fig. 3A). WR132/A13L was non-reactive with VIG, although was reactive with sera from naïve individuals undergoing a primary infection (Supplemental Data, Fig. 3B). Other assay parameters, such as blocking buffer used, incubation times, and development system used, were all determined using a systematic approach to yield optimum assay performance. During data acquisition using the ELISA assay, a standard operating procedure was adhered to throughout, and replicates wells of the same sera samples in different plates were used for data normalization.

Overall, the ELISA data correlated well with signal intensities by array. Shown in Supplemental Data, Fig. 4 are longitudinal responses to WR148 by 8 vaccinia naïve individuals in protocol #1 given Dryvax (compare with Fig. 1A). By ELISA (panel A), IgG was first detected on d14, although titers were higher and responses more prevalent among the donor at d28. The WR148 data mirror closely the array data (panel B) and show a high correlation coefficient ($R^2=0.891$; panel C). Correlation coefficients for other antigens were: WR101/H3L=0.823, WR132/A13L=0.621, WR070/I1L=0.599 and WR118/D13L=0.594. WR148 gave excellent signal to background ratio (d0 vs d28) and the largest number of seropositives.

Shown in Fig. 5 are compiled data from all 6 recombinant purified antigens used in ELISA to assay sera from individual human volunteers before and after smallpox vaccination. Fig. 5A shows vaccinia-naïve individuals from protocol #3 ([62] also shown in Supplemental Data, Fig. 1), before and after Dryvax inoculation. Under the conditions used, WR148 and WR113/D8L gave the strongest OD_{450nm} signals overall and were also the most commonly recognized antigens, giving 95.7% and 97.9% coverage of the Dryvax vaccinees, respectively. The frequency of recognition of the remaining 5 antigens varied individually from 21.3 to 74.5%. The weakest reactivity was seen against WR070/I1L. 'Multiplexing' WR113/D8L with WR148 (by counting a response to either one or both antigens as a seropositive) did not increase coverage above that achieved by WR113/D8L alone. Combining 2 or more of the remaining 5 antigens increased coverage above the individual antigens, owing to partially overlapping seroreactivity profiles. For example, combining WR118/D13L and WR132/A13L provided 89.4% coverage, compared to 63.8% and 74.5% individually. However, none of these matched the performance of WR113/D8L alone. We also detect reactivity in a small number of naïve individuals on d0, similar to that seen using the arrays, with a good correlation between the two assay systems ($R^2=0.64$). For this reason, multiplexing tended also to increase the rate of seropositivity in the d0 samples. None of the naïve individuals enrolled into this particular trial were known to have been

previously vaccinated according to the inclusion/exclusion criteria for the study. The etiology and significance of these antibodies is currently unknown.

For comparison, sera from 25 individuals vaccinated previously that received a second Dryvax inoculation are shown in Fig. 5B. Consistent with their prior exposure is elevated seroreactivity to several antigens on d0 compared to the naïve population. This was detected in less than a third of the previously vaccinated group, and several of the seropositives were close to the cutoff; both observations are consistent with waning titers of antibody. WR101/H3L and WR118/D13L appear to be the most sensitive to previous vaccination with 32% and 24% coverage, compared to 6.7% and 4.4% in naïve individuals, respectively. Combined, these two antigens provided 48% coverage compared to 8.9% in naïves. After the boost, stronger signals relative to naïves were seen against most antigens (with the striking exception of WR132/A13L) and an increased frequency of recognition, both of which are consistent of an anamnestic memory response. This boosting effect is most dramatic for the WR070/I1L antigen, where the response in a primary infection was weakest. Reciprocally, there was virtually no boosting of titers to WR132/A13L after the second inoculation, despite a robust primary response, which raises interesting questions about the memory pool to this antigen. The ratio of response to WR132/A13L and WR070/I1L using this ELISA may have some utility for distinguishing primary and secondary responses to Dryvax.

For comparison with Dryvax, in Fig 5C are shown naïve MVA vaccinees from protocol #1 (same trial featured in Fig. 1A). There was ~5% seroreactivity to WR148, both before and after MVA vaccination as anticipated. (This can be reduced to zero by raising the cutoff to Avg+3SD of d0 signals). WR113/D8L provided the most robust response of all the antigens tested, with 93.8% coverage of MVA vaccinees (at both Avg+2SD and 3SD cutoffs). WR101/H3L and WR118/D13L were the next most commonly recognized antigens at 66.7% and 41.7%, respectively, which is increased to 81.3% when data for these two are combined. However, multiplexing also increased seroreactivity on d0, from 6.3% individually to 10.4% when combined. Combining WR113/D8L with WR101/H3L and WR118/D13L provides 100% coverage of MVA vaccinees, but seroreactivity on d0 also increased to 14.6%.

We also used the ELISAs to measure responses to MVA in individuals vaccinated previously against smallpox (protocol #6). Responses to WR118/D13L, WR101/H3L, and WR070/I1L were elevated on d42 compared to d0, and the numbers of responders increased compared to naïve MVA vaccinees; responses to WR148 and WR132/A13L remained unchanged after boosting (data not shown). These data are consistent with array data and indicate heterologous boosting by MVA of pre-existing immunity to vaccinia.

We also examined the effect of multiplexing using receiver operator characteristics (ROC) analyses from the ELISA data from the same data set from naïve MVA and Dryvax vaccinees (Fig. 6). The rates of detection of the true positive (sensitivity) and false positives (1-specificity) were determined at different cutoff thresholds, and the antigens ranked by the area under the curve (AUC). For Dryvax, the antigen that best discriminates naïve from vaccinated was WR113/D8L, followed by WR148 then WR118/D13L. WR070/I1L was the weakest discriminator. For MVA, the best discriminator was again WR113/D8L, followed by WR101/H3L then WR118/D13L. Both WR148 and WR070/I1L showed no discriminatory power after MVA vaccination. Small improvements in sensitivity and specificity of WR113/D8L were obtained by combining with additional antigens with the next highest AUCs. Overall, these data correspond well with the frequency of recognition analysis discussed above in Fig. 5.

Discussion

Antibodies are known to represent a major component in protection generated by vaccinia [71, 72], and the measurement of antibodies to vaccinia is a convenient means for monitoring immune status. Although smallpox has been eradicated and routine vaccination has been discontinued, the potential for the intentional release of smallpox or the emergence of monkeypox in human populations [73] may require vaccination and monitoring of the general population. MVA is also used as a delivery vehicle for heterologous antigens and it is therefore of importance to vaccine developers to have means for measuring a response to the vehicle as well as the heterologous cargo. Currently, the standard methods for measuring the antibody response are the ELISA, which uses infected cell lysates as detection antigen, and the plaque reduction neutralization test (PRNT). The gold standard is the PRNT, a functional bioassay that measures the virus-neutralizing activity of serum *in vitro* and may provide a close approximation to protection *in vivo*. The PRNT is time-consuming and requires specialized equipment. A more precise understanding of the relative importance of individual antigens in the immune response will come from developing recombinant protein assays. We had previously used proteome microarrays to identify suitable vaccinia antigens for diagnostic use and for subunit vaccines. In one study we compared the antibody profiles of MVA and Dryvax as a way of comparing their immunogenicity [35]. While there were differences, we concluded the profiles were comparable, particularly with respect to responses to membrane proteins that might be expected to mediate virus neutralization. The study reported herein confirms and expands on this, with a larger sera collection and with an array using a recloned ORFeome library. We have also used more rigorous statistical treatments to identify significant differences between the profiles and to prioritize antigens for the development of immunoassays (ELISAs) that could be used to monitor vaccination.

As a result of array screening, we expressed and purified 6 proteins from VACV-WR in *E. coli* and tested them in ELISAs. Five of these antigens (WR070/I1L, WR101/H3L, WR113/D8L, WR118/D13L, and WR132/A13L) are dominant in both Dryvax and MVA profiles, while WR148 was included because of its dominance in Dryvax only. In the context of vaccinia serology, it is conventional to focus on membrane proteins. While this is a proven approach for antibody-mediated protection and subunit vaccine design [74–76], the choice of ideal antigens for serodiagnostic applications seems less dependent on membrane proteins. Three of the 6 antigens we selected are surface proteins from the IMV (intracellular mature virion) stage [45–47], the most abundant and robust form of the virion and is believed to mediate host-to-host transmission [77, 78]. WR101/H3L and WR113/D8L are 37.4 kDa and 35.4 kDa integral IMV membrane proteins that mediate bind host cell attachment by binding heparan sulfate [79, 80] and chondroitin sulfate [81–83], respectively. Both proteins are also important targets of neutralizing antibodies, and when administered to mice as subunit vaccines, both antigens can protect against lethal orthopoxvirus challenge [76, 84, 85]. The third, WR132/A13L, is a small 7.7 kDa type II integral membrane protein that is abundant in the IMV [86, 87]. Only 3 amino acids are predicted to be exposed at the virion surface and it is not known to play a major role in antibody-mediated neutralization. The remaining three proteins we tested in the ELISA are also present in the virion: WR070/I1L is a DNA-binding capsid protein [88], and a relatively minor component of the virion mass [45, 46]. The remaining two, WR148 and WR118/D13L, are present in mass spectra derived from virions, although appear to function primarily in the cytoplasm of host cells. WR118/D13L assists with virion assembly in virus factories by forming a curved honeycomb scaffold on the exterior of immature virions [89]. WR148 is a 94kDa truncated homolog of the cowpox A-type inclusion (ATI) protein. The ATI protein normally forms protein-dense cytoplasmic inclusions that capture IMVs (a process called occlusion), which may serve to protect IMVs from the extracellular environment [90]. The truncated ATI mutant in vaccinia does not form such inclusions, and its function is still being elucidated

[91]. Together these 6 antigens were screened against sera from clinical trials of Dryvax and MVA. A less abundant form of the vaccinia virion is the EEV (extracellular enveloped virion), a double enveloped particle that is more labile than IMVs and thought to mediate cell-to-cell transmission within the infected host [77, 78]. EEVs have four membrane proteins that are not found on IMVs: WR052/F13L, WR187/B5R, WR181/A56R and WR156/A33R. Although none of these were represented in the ELISAs reported here (due to manufacturing considerations), the array data presented indicate responses to WR052/F13L and WR187/B5R are equally strong in MVA and Dryvax vaccinees, whereas responses to WR181/A56R and WR156/A33R are significantly stronger in Dryvax vaccinees and may be discriminatory.

In the ELISA, one of the best diagnostic antigens for Dryvax is the ATI homolog, WR148. This antigen is also recognized strongly by all other orthopoxvirus immune sera we have tested, including MPVX, VARV, and VACV-Lister ([34] and unpublished). Another excellent serodiagnostic antigen is the IMV membrane protein WR113/D8L, which is recognized by virtually all Dryvax and MVA vaccinees. WR113/D8L is the best single antigen to provide coverage of MVA vaccinees, with slight increases in coverage when combined with WR101/H3L and WR118/D13L. MVA diagnostic antigens are shared with Dryvax, in that the dominant antigens in MVA are a subset of those in the Dryvax profile. An unexpected result was the finding in the ELISA that WR132/A13L was widely recognized by naive individuals undergoing a primary response to Dryvax, but was not recognized by individuals undergoing a secondary response, or by vaccinia immune globulin (VIG, a pooled IgG derived from hyperimmune sera). This differential recognition is also seen using the microarray platform (data not shown). The basis for this striking pattern of reactivity is not known, but may be related to the properties of the protein itself and the way it is exposed to the immune system. Interestingly, we saw a reciprocal situation with WR070/I1L, which was only recognized after Dryvax re-vaccination. Neither antigen is recognized well by MVA vaccinees in the ELISA. This study adds to a growing number of reports that have used recombinant proteins in place of whole virions for ELISAs. These antigens include the EEV membrane proteins WR156/A33R [92–94], WR157/A34R [93], WR159/A56R [92, 93] and WR187/B5R [92–95] (all produced in eukaryotic expression systems), and IMV membrane proteins WR088/L1R [92, 94], WR101/H3L [92] and WR150/A27L [92]. The data presented herein indicate that excellent sensitivity and specificity can also be achieved with both membrane and non-membrane proteins expressed *E. coli*.

Vaccination of individuals with pre-existing immunity to vaccinia is known to be associated with an anamnestic antibody response, characterized by elevated titers compared to a primary infection [13, 61, 96]. Re-vaccination is also associated with attenuation of the skin take, lower take rates, and a reduced incidence of fever and viral shedding [13, 97]. Thus, there have been concerns that pre-existing immunity to vaccinia may interfere with the use of MVA in the population to heterologous antigens delivered by recombinant MVA. Although not examined directly here, our data do indicate pre-existing immunity does not interfere with the antigenicity of the MVA vector itself. Responses to some antigens were higher in the previously vaccinated group (e.g., WR070/I1L and WR150/A27L) and others (notably WR187/B5R) were lower in the previously vaccinated group. Thus, any attenuation or enhancement of MVA antigens caused by previous vaccination appears to vary between different antigens. Moreover, the presence of pre-existing antibodies that may block replication may not have as large an effect on MVA, which is given as a bolus injection, as they would on a recombinant replication competent vector that is more reliant on replication for expression. In a reciprocal situation, pre-existing MVA immunity to shared (orthologous) antigens is boosted by Dryvax whereas the primary response to the non-

orthologous WR148 appears reduced. Screening these samples by the ELISAs developed here is underway as this may provide improved sensitivity compared to the array format.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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***Highlights**

- > Vaccinia antibodies were profiled before and after MVA and DVX vaccination.
- > As a result, WR148, D8L, D13L, H3L, A26L and I1L were purified and evaluated in ELISAs.
- > The membrane protein, D8L, provided the best sensitivity and specificity for monitoring both vaccines.
- > The A-type inclusion protein homolog, WR148, provided the best discrimination.
- > A13L/I1L ratio discriminated primary and secondary responses to DVX.

Fig. 1. Dynamics of MVA and Dryvax antibody profiles in vaccinia naive donors
Antigens from vaccinia virus strain Western Reserve (VACV-WR) were printed on microarrays and probed with sera from groups of MVA and Dryvax vaccinees. Nomenclature for antigens is WR number/ Copenhagen strain ortholog. The plots show the average raw signal intensity $\pm$ SEM of each antigen for each vaccination group. Day 0 signals were subtracted from all time points. The reactive antigens are ranked in descending order independently for each group. **A:** MVA given on d0 and d28 (group 1a, n=25); **B:** Dryvax given on d0 (group 2, n=8); A and B are longitudinal sera from a single representative protocol (protocol #1). **C:** Dryvax given on d0 from protocol #2 (n=10) [61].

Fig. 2. Discriminatory and cross-reactive antigens in MVA and Dryvax profiles

Peak MVA and Dryvax antibody profiles from protocol #1 (groups 1a+1b and group 2, respectively) were defined by microarray and compared by t test. Histograms are plotted from raw signal intensities subtracted of d0 signals, and ranked by the Dryvax signals. The overlaid p-values were determined from log transformed and normalized signals at the peak subtracted of d0. Antigens are segregated into differentially and non-differentially reactive according to Benjamini-Hochberg corrected p-value (p_{BH}) for each reactive antigen ($<$ or ≥ 0.05 , respectively).

Fig. 3. Heterologous boosting by MVA in individuals vaccinated previously against smallpox Individuals known to have been vaccinated previously against smallpox were given boosts of MVA or Dryvax, and sera probed against VACV-WR proteome microarrays (**A**, **B**) or were vaccinia naïve (**C**, **D**). The ratio of post/pre signal intensities for each antigen are shown to the right of each graph. The values shown in each panel are raw signal intensities without d0 subtraction, and antigens are ranked separately by the profile prior to boosting. The position of the WR148 response is highlighted by the asterisk (*). **A**: MVA arm from protocol #6 (n=25). Individuals were inoculated twice (d0/d28) and profiles on d0 ('pre') and d56 ('post') plotted. **B**: Dryvax arm from protocol #5 (n=25). Individuals were inoculated on d0, and profiles on d0 ('pre') and d28 ('post') plotted. Protocol #5 examined revaccination of individuals with 3 different dilutions of Dryvax (1/10, 1/5 and undiluted). All individuals showed a take to the inoculation. A three-way T test analysis revealed no significant different differences between the profiles engendered by the three different doses (data not shown), thus the data could be pooled for this figure. **C**: Corresponding MVA profile in vaccinia naïve individuals (group 1a from protocol #1, n=25). **D**: Corresponding Dryvax profile in vaccinia naïve individuals (group 2 from protocol #1, n=8).

Fig. 4. Pre-existing immunity to MVA does not block response to 'heterologous' antigen in Dryvax

Three groups of vaccinia-naïve individuals from protocol #4 [63–65] were given vaccinations as follows. **A:** MVA on d0 and d28, followed by Dryvax on d112 (group 2). **B:** MVA on d0 and d28, followed by placebo on d112 (Group 3). **C:** Dryvax on d112 (group 4). Proteome-wide antibody profiles were determined at regular time points by microarrays; only responses to WR148 (present in Dryvax only), WR118/D13 and WR113/D8 are shown.

Fig. 5. ELISA data using sera from Dryvax and MVA vaccinees using 6 different purified VACV-WR antigens

A: Protocol #3 [62], vaccinia naïve individuals (n=47) before and 28d after Dryvax. **B:** Protocol #5, previously vaccinated individuals (n=25) before and 28d after Dryvax revaccination. **C:** Protocol #1, vaccinia naïve individuals (n=48) before and 42d after two MVA inoculations in d0 and d28. Horizontal line represents cutoff, above which a serum was considered seropositive. Figures in the top left of each panel indicate the percentages of seropositive samples before/after vaccination. The cutoff was defined the average+2SD of the pre-vaccination (d0) signals using log transformed data. To help evaluate pre-existing immunity in the previously vaccinated individuals before revaccination (d0 in panel B), the cutoff was defined from the vaccinia naïve population.

Fig. 6. ROC curves of ELISA data using 6 different purified VACV-WR antigens
 ELISA data for vaccinia naïve individuals given Dryvax or MVA (described in Fig 5) were subjected to receiver operator characteristic (ROC) analysis to find antigens that discriminate between profiles on d0 and at the peak response (d28 for Dryvax and d42 for MVA). The best discriminators are antigens with an area under the curve (AUC) approaching 1.0. An antigen with no discriminatory power would have an AUC of 0.5 (represented by the diagonal). **A:** Dryvax, single antigens; **B:** Dryvax, multi-antigen analysis; **C:** MVA, single antigens; **D:** MVA, multi-antigen analysis; **E:** AUC values for single and multi-antigen analyses, ranked by AUC of the Dryvax response.

Table 1

Frequency of recognition of VACV-WR antigens by vaccinia naïve individuals after MVA and Dryvax vaccination.

Microarray Antigen (VACV-WR)	Name	Percent seropositive	
		MVA (n=88)	DVX (n=68)
118/D13L	Virion assembly protein	53.9	97.1
101/H3L	IMV heparin binding surface protein	79.8	92.6
113/D8L	IMV membrane protein	88.8	91.2
150/A27L	IMV surface protein	5.6	91.2
130/A11R	Membrane formation protein	24.7	83.8
132/A13L	IMV membrane protein	23.6	79.4
137/A17L	IMV membrane protein	14.6	76.5
148/-	A-type inclusion protein homolog	3.4	75.0
156/A33R	EEV membrane phosphoglycoprotein	18.0	73.5
129/A10L	Core protein 4a precursor	51.7	72.1
070/I1L	DNA-binding core protein	12.4	70.6
103/H5R	Late gene transcription factor VLTF-4	16.9	66.2
187/B5R	EEV type-1 membrane glycoprotein	67.4	60.3
056/F17R	Putative DNA-binding phosphoprotein	30.3	52.9
091/L4R	Core protein vp8	7.9	48.5
181/A56R	EEV membrane hemagglutinin	16.9	45.6
157/A34R	C-type lectin-like EEV protein	44.9	39.7
052/F13L	Palmytilated EEV membrane protein	32.6	35.3
010/C10L	IL-1 Receptor antagonist	5.6	33.8
133/A14L	Phosphorylated IMV membrane protein	39.3	30.9
046/F7L	?	13.5	26.5
079/G3L	?	7.9	25.0
149/A26L	P4c precursor	4.5	25.0
069/O2L	Glutaredoxin 1	2.2	25.0
172/A46R	Toll-IL1-receptor [TIR]-like	18.0	23.5
104/H6R	Topoisomerase type I	5.6	22.1
178/A52R	Intracellular TLR and IL-1 signaling inhibitor	5.6	20.6

Sera from MVA and Dryvax (DVX) arms of several independent clinical trials were probed against VACV-WR proteome microarrays. Array data were log transformed and normalized, and T test comparisons made of the antibody profiles of the different MVA and DVX arms. Arms that were not significantly different ($p_{BH} \geq 0.05$) were pooled. MVA data were pooled from MVA arms of protocols 1+4, and DVX data were pooled from DVX arms of protocols 1+2+3 (see Supplemental Data Table 1). Values in the table are the % seropositives for each antigen in each vaccine group. These were determined at the peak of the response, i.e. day 42 for MVA and day 28 for Dryvax, using the log transformed data. The cut off for positivity was defined as the average signal intensity+2SD on day 0. Antigens are ranked in descending order of the Dryvax group. (For brevity, only the antigens recognized by over 20% of the Dryvax vaccinees are listed).