

# Redundancy and Plasticity of Neutralizing Antibody Responses Are Cornerstone Attributes of the Human Immune Response to the Smallpox Vaccine<sup>∇†</sup>

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**The smallpox vaccine is widely considered the gold standard for human vaccines, yet the key antibody targets in humans remain unclear. We endeavored to identify a stereotypic, dominant, mature virion (MV) neutralizing antibody target in humans which could be used as a diagnostic serological marker of protective humoral immunity induced by the smallpox vaccine (vaccinia virus [VACV]). We have instead found that diversity is a defining characteristic of the human antibody response to the smallpox vaccine. We show that H3 is the most immunodominant VACV neutralizing antibody target, as determined by correlation analysis of immunoglobulin G (IgG) specificities to MV neutralizing antibody titers. It was determined that purified human anti-H3 IgG is sufficient for neutralization of VACV; however, depletion or blockade of anti-H3 antibodies revealed no significant reduction in neutralization activity, showing anti-H3 IgG is not required in vaccinated humans (or mice) for neutralization of MV. Comparable results were obtained for human (and mouse) anti-L1 IgG and even for anti-H3 and anti-L1 IgG in combination. In addition to H3 and L1, human antibody responses to D8, A27, D13, and A14 exhibited statistically significant correlations with virus neutralization. Altogether, these data indicate the smallpox vaccine succeeds in generating strong neutralizing antibody responses not by eliciting a stereotypic response to a single key antigen but instead by driving development of neutralizing antibodies to multiple viral proteins, resulting in a “safety net” of highly redundant neutralizing antibody responses, the specificities of which can vary from individual to individual. We propose that this is a fundamental attribute of the smallpox vaccine.**

Smallpox is an exceptionally lethal disease ( $\leq 30\%$  mortality) caused by variola virus. Smallpox is highly infectious and is thought to be transmitted both as an aerosol and via fomites, causing a lung infection that subsequently spreads during a viremic phase, leading to the classic disseminated rash or “pocks” (16, 22, 23). Smallpox was endemic throughout Europe, Africa, Asia, and the Indian subcontinent throughout most of the last 2,000-plus years. Given the high mortality rate of smallpox infections in humans and the endemic nature of the disease, smallpox may have caused more human deaths during the past two millennia than any other single disease (27). While wild smallpox no longer exists, variola virus is a potential bioterrorism agent of great concern (3, 4, 23, 44).

Vaccines are one of the most cost-effective medical treatments in modern civilization (47). A smallpox vaccine was the first human vaccine, and the modern smallpox vaccine, live vaccinia virus (VACV), is the most successful human vaccine,

bringing about the worldwide eradication of smallpox disease due to a heroic World Health Organization campaign in the 1960s and 1970s (16). The smallpox vaccine is generally considered the gold standard for vaccines, and elucidating the immunobiology underlying the protection provided by the smallpox vaccine will continue to reveal vaccinology principles that can be applied to future vaccine development against other infectious scourges.

With animal models (6, 15, 18, 36) and in human clinical studies (28, 37, 53, 60), there are substantial data demonstrating the important role of antibodies in protecting against smallpox and related poxviruses (5). The most compelling animal model data came from a recent monkeypox study with macaques, demonstrating that smallpox vaccine-induced antibody responses are both necessary and sufficient for protection against lethal monkeypox infection while memory T cells were dispensable (15). In humans, high neutralizing antibody titers have been associated with protective immunity against smallpox infection (37, 53). Long-term antibody titers and T-cell memory for the smallpox vaccine do not correlate in humans (11, 19), excluding the possibility that antibody titers were simply a biomarker for memory T cells. Vaccinia immune globulin (VIG) is an effective treatment against smallpox (28), since it was able to reduce the number of smallpox cases  $\sim 80\%$

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among exposed individuals in four case-controlled studies (25, 28, 32, 33, 38).

Neutralizing antibodies mainly confer protection through the recognition of structures on the surface of virus particles, and therefore, antiviral antibodies directed against the surface of virions are of primary interest. Poxviruses (vaccinia, variola/smallpox, and monkeypox) have two virion forms, intracellular mature virions (MV) and extracellular enveloped virions (EV), each with distinct biology (10, 55). As such, an understanding of the virion structures is required to develop knowledge regarding the targets of protective antibodies. MV and EV virion forms express mutually exclusive sets of viral proteins on the surface (10, 40, 55). The most abundant particle is the MV, which accumulates in infected cells and is released as cells die (40). The relative roles of antibodies against MV and EV in protective immunity still remain unclear, but a working hypothesis is that antibodies against MV surface antigens might be expected to play a major role in preventing host-to-host transmission, given that MVs are environmentally stable infectious particles and may represent the principle type involved in transmission between hosts (5, 17); antibodies against EV targets may play a major role in preventing viral spread within an infected host. Animal model studies have clearly shown that antibodies against either the MV or EV virion form can be protective (13, 18, 36, 46).

The focus of the study reported here is the understanding of the key MV surface protein target(s) of the human neutralizing antibody response to the smallpox vaccine. While the smallpox vaccine elicits a potent neutralizing antibody response in humans (conventionally measuring MV-specific neutralization) (5, 16), the key antibody targets remain unclear. In 2005, at the initiation of this study, there were 17 known or predicted MV surface proteins. With use of various animal models, neutralizing antibodies directed against five of these proteins have been found: L1, A27, A17, H3, and D8 (1, 2, 7, 14, 29–31, 34, 35, 46, 50, 51, 59, 61). Nevertheless, only one of these targets (H3) had been directly demonstrated to be the target of neutralizing antibodies in humans immunized with the smallpox vaccine (13).

We set out to find a single stereotypic diagnostic serological marker of protective humoral immunity induced by the smallpox vaccine to understand the nature of why the smallpox vaccine is so effective. This would be an important advance in our understanding of the protective mechanism of the smallpox vaccine and may help reveal general principles of the nature of protective/neutralizing antibody responses to large viral (or other) pathogens consisting of numerous surface antigens. What we have discovered, using multiple experimental approaches, is that redundancy and diversity are defining characteristics of the human antibody response to the smallpox vaccine.

#### MATERIALS AND METHODS

**Sera.** Human plasma samples from a cohort of 58 volunteers were stored as aliquots at  $-80^{\circ}\text{C}$ . Eight unvaccinated donors and 50 Dryvax-immunized donors were used. Plasma samples were obtained from 14 days to 40 years postvaccination. Approximately 50% of samples were obtained after primary immunization, and 50% were obtained after secondary/booster immunization. Rabbit anti-H3 serum was obtained by immunizing two rabbits with the recombinant H3 protein (rH3) (200  $\mu\text{g}$  rH3/dose) emulsified in Freund's complete adjuvant and boosting seven times over 12 weeks with antigen in incomplete Freund's adjuvant. Rabbit-

anti L1 serum was generated by immunizing two rabbits with recombinant L1 protein (200  $\mu\text{g}$  recombinant L1 (rL1)/dose) emulsified in Freund's complete adjuvant and boosting three times at weeks 3, 6, and 10 with antigen (100  $\mu\text{g}$  rL1/dose) in incomplete Freund's adjuvant (ProSci Incorporated, Poway, CA). Mouse anti-vaccinia virus serum was obtained from C57BL/6J mice (Jackson Laboratory) 45 days post-infection with  $2 \times 10^5$  PFU total VACV Western Reserve strain (VACV<sub>WR</sub>) administered intraperitoneally. Human, mouse, and rabbit sera were heat inactivated prior to use ( $56^{\circ}\text{C}$ ; 30, 30, and 60 min, respectively). Human VIG (Cangene Corp., Winnipeg, Canada) was stored at  $-80^{\circ}\text{C}$ .

**Viruses.** VACV<sub>WR</sub> stocks were grown on HeLa cells in D-10 (Dulbecco's modified Eagle medium [DMEM] plus 10% fetal calf serum [FCS] plus penicillin/streptomycin/glutamine) in T175 flasks (Falcon; Becton Dickinson), infecting at a multiplicity of infection of 0.1 to 0.5. Cells were harvested at 2.5 to 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  $-80^{\circ}\text{C}$  as virus stock. Titers of VACV<sub>WR</sub> stocks were determined with VeroE6 cells ( $\sim 2 \times 10^8$  PFU/ml). VACV New York City Board of Health (VACV<sub>NYBOH</sub>) stocks were generated as low-passage stocks from commercial Dryvax, using the same conditions as those for VACV<sub>WR</sub> described above. Purified VACV<sub>WR</sub> stocks were made via centrifugation through a sucrose cushion. Standard VACV<sub>WR</sub> stock virus was sonicated (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<sub>2</sub>) (9). VACV<sub>WR</sub> was centrifuged (SW28 rotor) at 13,500 rpm (33,000  $\times$  g) for 80 min at  $4^{\circ}\text{C}$ . The VACV<sub>WR</sub> 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<sub>WR</sub> was stored at  $-80^{\circ}\text{C}$ . The UV-inactivated vaccinia virus (UV-VACV) was prepared as described previously (11, 58).

**VACV neutralization assays.** VeroE6 cells were seeded at  $1.5 \times 10^5$  cells/well into 24-well Costar plates (Corning, Inc., Corning, NY) and used the following day (75 to 90% confluence). Serum or plasma samples were treated as described below (blockade, reverse immunoprecipitation, purification, etc.) and then incubated with 50  $\mu\text{l}$  of freshly sonicated VACV<sub>WR</sub> ( $10^4$  PFU/ml) overnight at  $37^{\circ}\text{C}$  with 5% CO<sub>2</sub> according to the method of Newman et al. (41). Negative control sera from unvaccinated humans, mouse sera, and rabbit sera were treated under the same conditions. Multiple wells of VACV<sub>WR</sub>-alone controls were always used. Medium from 24-well plate wells was aspirated, and samples were added and allowed to adsorb for 60 min at  $37^{\circ}\text{C}$ . Cells were rinsed with warm phosphate-buffered saline (PBS). One milliliter of D-10 medium was then added and the plates incubated for 40 to 48 h. Cells were fixed and stained with 0.1% crystal violet in 20% reagent alcohol (90% ethanol, 5% methanol, 5% isopropanol), and plaques were quantified. Two methods were used to quantify VACV neutralizing antibodies. First, a conventional 50% plaque reduction number titer (PRNT<sub>50</sub>) approach was used, where the antibody sample of interest was serially diluted  $1.5\times$  prior to incubation with VACV. The neutralization titer (PRNT<sub>50</sub>) was defined as the reciprocal of the last dilution of the plasma that reduced the average number of plaques by  $\geq 50\%$  compared to the mean number of VACV-alone plaques. The second approach was to quantitate VACV neutralization at a single predefined plasma/antibody sample dilution, wherein the percentage of VACV neutralization was defined according to the following formula:  $[(\text{PNV} - \text{PNS})/\text{PNV}] \times 100$ , where PNV was the average number of plaques for VACV<sub>WR</sub> alone and PNS was the average number of plaques in the presence of each experimental sample. Treated versus untreated samples were then compared to obtain the percentage change in neutralization activity ("VACV neutralization, percentage of starting levels"). Data sets completed over the course of multiple experiments on multiple days were normalized to a standard.

**Blockade of specific antibodies in human plasma.** Human smallpox vaccinee plasma samples (10  $\mu\text{l}$ ) were preincubated with the rH3 or rL1 protein (0, 0.1, 1, 10, or 50  $\mu\text{g}$ ) or with  $10^7$  PFU of UV-VACV for 2 h at room temperature in a 50- $\mu\text{l}$  total volume. Samples were then used in VACV neutralization assays. Viral recombinant protein alone incubated with VACV was used as a negative control. Mouse (1:5 final dilution) and rabbit (1:250 final dilution) serum samples were treated comparably. In the case of UV-VACV, the average number of plaques for the UV-VACV/VACV mixture alone was used.

**Reverse immunoprecipitation.** One hundred microliters of human plasma, mouse sera, or rabbit sera were preincubated with the rH3 or rL1 protein (0 or 10  $\mu\text{g}$ ) for 2 h at room temperature in a 1.5-ml microcentrifuge on rotator in an equal volume of binding buffer (PBS [1 $\times$ ], 40 mM imidazole [pH 7.4]), followed by addition of 50  $\mu\text{l}$  nickel-Sepharose (Ni-Sepharose) (Amersham Biosciences GE Healthcare, Uppsala, Sweden) and incubation for one additional hour. Samples were reverse immunoprecipitated by centrifugation (500  $\times$  g, 5 min). The supernatant was collected and used in VACV neutralization assays. The

depletion was also performed in reverse order by preincubating the recombinant protein with Ni-Sepharose and then adding the plasma sample. Each sample was processed as described above.

**Affinity chromatography and purification of specific antibodies in human plasma.** The HiTrap NHS-activated column (Amersham Biosciences GE Healthcare, Uppsala Sweden) comprises *N*-hydroxy-succinimide ester attached to Sepharose, which can react with primary amino groups to give a stable covalent amide linkage. The desired ligand (rH3 or rL1) was dissolved in coupling buffer (0.2 M NaHCO<sub>3</sub>, 0.5 M NaCl, pH 8.3) and buffer exchanged twice (10,000-molecular-weight cutoff, 15 ml Amicon Ultra; Millipore Corporation, MA) before suspension at 0.5 or 1.0 mg/ml. Preparation of the column was done according to the manufacturer's protocol (Amersham Biosciences GE Healthcare, Uppsala, Sweden). Coupling of 0.5 mg or 1.0 mg rH3 or rL1 to the column was performed at room temperature for 30 min, recirculating the protein solution using a peristaltic pump (0.5 ml/min). Excess amino groups were then deactivated with blocking buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3), and the column was extensively washed with 0.1 M acetate–0.5 M NaCl (pH 4). The column was stored at 4°C in storage solution (0.05 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1% NaN<sub>3</sub>, pH 7.0). The efficiency of protein binding was calculated by H3 or L1 enzyme-linked immunosorbent assay (ELISA), taking rH3 (or rL1) samples before and after column binding and using that material to coat ELISA wells. Given the known concentration of rH3 prior to column binding, serial dilutions of H3-reactive mouse serum could be used to calculate the concentration of the residual rH3 not bound to the column. Column binding of rH3 or rL1 was determined to be greater than 99% efficient.

Plasma from single donors was diluted 1:5 in binding buffer (75 mM Tris-HCl, pH 8.3, final) and filtered (0.45 μm) before application to the rH3 or rL1 coupled affinity column. The column was prepared by washing with binding buffer. Ten to twenty milliliters of plasma (50 to 100 ml total volume after dilution) was applied to the column at 0.5 ml/min. Flowthrough fractions were collected. The column was extensively washed prior to elution steps. Specific anti-H3 antibodies were eluted using 100 mM glycine (pH 2.5). One-milliliter fractions were eluted directly into microcentrifuge tubes containing 70 μl of 1 M Tris-HCl (pH 9.0) to neutralize the pH. Anti-L1 antibodies were eluted using pH 2.5 glycine or 3 M guanidine (pH 7.4). Columns were stripped and washed between uses and stored at 4°C in 1 ml of storage buffer. Anti-L1 antibodies eluted in guanidine were buffer exchanged using a protein-desalting column (Zeba desalt spin column) according to the manufacturer's protocol (Pierce, IL).

**Protein synthesis and proteome arrays.** Sixteen-pad nitrocellulose FAST slides (Whatman, Florham Park, NJ) were used for protein microarray printing. Proteins were printed at Scripps (TSRI) using a custom arrayer with 100-μm pins built by Robotic Labware Design (RLD). Humidity was maintained at 40 to 60% during printing. Microarray slides were subsequently dried and stored in a desiccator at –80°C. Most VACV proteins printed were generated by rapid translation system (RTS; Roche) in vitro synthesis. Vaccinia genes were cloned into pXi (pNHisCHA derived) (13) and sequenced prior to protein synthesis using the Roche RTS *Escherichia coli* in vitro coupled transcription-and-translation expression system. RTS reactions without a plasmid were used as negative controls. Expression was confirmed by dot blot, Western blot, or microarray probing for His tags. Whole-virus antigen was printed using 10-fold-concentrated, PBS buffer-exchanged, psoralen-inactivated (13) standard VACV<sub>WR</sub> stock. Uninfected HeLa cell lysate, processed identically, served as a negative control for VACV<sub>WR</sub> virus. The purified His-tagged H3 protein (13) was printed at 100 μg/ml. *E. coli*-produced H3, L1, F9, A21, and A28 were purified and refolded under oxidizing conditions to generate natively folded protein with appropriate disulfide bond formation (56). All antigens were resuspended in 0.02% Tween 20 prior to printing.

Human samples were initially screened using a large protein microarray covering 185 VACV proteins (13). The majority of experiments done in this study were done using a compact microarray (see Fig. 1) consisting of 55 VACV proteins identified as an antibody target in any species, as well as all known virion surface proteins (as of 2005). This strategy allowed for an 8-fold increase in the numbers of replicate experiments (16-pad microarray slide versus 2-pad microarray slide).

Samples were diluted in array blocking solution (protein array blocking solution; Whatman) and preincubated/preadsorbed for 30 min with 10% clarified *E. coli* lysate. (Clarified *E. coli* lysate was prepared using 100 mg/ml DH5α cells in PBS, sonicated, and then centrifuged at 6,000 × *g*. The lysate was then stored at –80°C.) After blocking, samples were added to microarray slides. Normal concentrations used were as follows: 1:50 mouse serum, 1:50 to 1:250 human plasma, or 1:125 to 1:2,500 rabbit serum. After 2 h of incubation at room temperature with constant agitation, slides were washed extensively in PBS supplemented with 0.05% Tween 20. Secondary antibody (Cy3-conjugated goat anti-mouse,

anti-human, or anti-rabbit immunoglobulin G [IgG] gamma chain Fc-region-specific Ig; Jackson ImmunoResearch) diluted 1:50 in array blocking solution was added for 1 h. Arrays were then washed extensively with PBS supplemented with 0.05% Tween 20 and PBS alone and then spun dry. Arrays were scanned on an Axon 400B GenePix scanner (Molecular Dynamics), and data were acquired using the GenePix Pro 5.1 software program. The total 532-nm fluorescence intensity of each spot was quantified as the signal strength. The background signal was subtracted using relevant matched control samples (e.g., RTS translation reaction without a plasmid or buffer alone), and the background-subtracted signal was converted to the final IgG relative units (RU) via a 10<sup>–6</sup>, 10<sup>–5</sup>, or 10<sup>–4</sup> transformation of all samples on a given microarray slide, depending on the microarray batch and GenePix settings used. Bar graph data are plotted as the average of duplicate protein prints (spots), with the full range shown as the error bar. Stringent signal thresholds (limit of detection) were established as 2 RU above the background level, which was more than 10 times the background observed in unvaccinated humans in almost all cases.

His-tagged rH3 was produced in *E. coli* (13) and purified to >95% purity before use in blocking and reverse immunoprecipitation. rH3 without the His tag was used for affinity column experiments. rL1 was produced as described previously (56). His-tagged rL1 was used for all L1 experiments.

**ELISA.** VACV, rH3, and rL1 ELISAs were carried out as described previously (13). Human IgG ELISAs were performed using the IgG standard (Southern Biotech, Birmingham, AL) on anti-human Ig (goat anti-human IgM plus IgG plus IgA; Caltag)-coated wells. After the calculation of the amount of IgG in each sample, the reciprocal of VACV neutralization specific activity using the PRNT<sub>50</sub> approach for each donor was determined (μg/ml of IgG necessary to neutralize 50% of VACV PFU).

**Statistical analysis.** Tests were performed using Prism 4.0 (GraphPad, San Diego, CA). Statistics were done using a two-tailed, unpaired *t* test with 95% confidence bounds unless otherwise indicated. *r*<sup>2</sup> values were calculated correlation coefficients. Data to be concurrently analyzed from multiple experiments were first normalized prior to statistical testing. Error bars for protein microarray ELISAs represent the full data range. Error bars for other graphs are ± one standard error of the mean (SEM) unless otherwise indicated.

## RESULTS

**Correlations with neutralizing antibody titers.** We hypothesized that the smallpox vaccine consistently elicits strong neutralizing antibody responses in a wide range of vaccinees because of the presence of an immunodominant neutralizing antibody target. We reasoned that we could identify this immunodominant neutralizing antibody target by correlating the levels of neutralizing antibody activity with levels of antibodies specific for each of the known MV surface proteins in a cohort of human vaccinees. The presence of an immunodominant neutralizing antibody target could be revealed as the antigen exhibiting the strongest correlation with neutralizing antibody titers. We examined this hypothesis using a VACV viral protein microarray-based approach, allowing a high-throughput strategy utilizing the microarrays to screen human plasma samples. Microarrays were printed with a set of ~50 VACV proteins (see Fig. S1A and B in the supplemental material), including 16 of the 17 MV surface proteins known when the study was initiated: A9, A13, A14, A17, A27, D8, D13, E10, H2, H3, I5, L5, L1, F9, A21, and A28. The 17th MV surface protein, A14.5, was not expressed, since it is small and highly hydrophobic, with only five amino acids predicted to be exposed on the surface. D13 was included as a potential surface protein, discussed in more detail later. All of the viral proteins were synthesized in *E. coli* or *E. coli* in vitro translation reactions (see Methods). While such an approach would be problematic for conventional eukaryotic surface proteins, which are normally glycosylated, *E. coli* expression was appropriate for studying MV surface proteins because they are not glycosylated (10). Proteins known to possess essential disulfide bonds (L1, A21, A28, and F9) were expressed in *E. coli* and refolded under

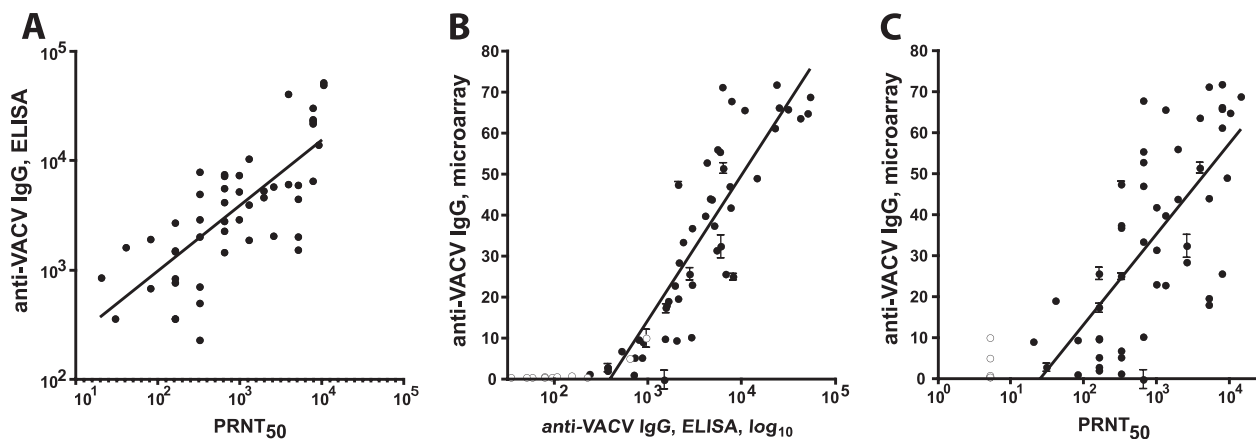


FIG. 1. Relationship between total anti-VACV IgG levels and virus neutralization activity. (A) Anti-VACV IgG levels in plasma samples from 50 smallpox vaccine recipients were quantified by endpoint dilution ELISA. Separately, VACV neutralizing antibody levels were quantified (PRNT<sub>50</sub>) using the same plasma samples. Data from each donor sample are plotted as anti-VACV IgG versus PRNT<sub>50</sub>. Linear regression analysis of the data set revealed a strong correlation ( $P < 0.001$ ); as to accuracy, the  $r^2$  value was 0.56. (B) Validation of accuracy of viral protein microarray measurement of human anti-VACV antibody responses. Relationship between human anti-VACV IgG titers as quantified by standard endpoint dilution ELISA versus microarray detection ( $P < 0.0001$ ;  $r^2 = 0.78$ ). (C) Relationship between anti-VACV IgG, measured by microarray, and neutralizing antibody titers ( $r^2 = 0.48$ ).

oxidizing conditions to produce natively folded protein. Non-disulfide-bonded forms of the L1 and F9 proteins were also made, but these exhibited no serological reactivity, indicating that correct folding was important for their immunogenicity (data not shown). Two additional MV surface proteins have since been identified, and these were not included in the synthesized microarrays (9, 40, 42, 43, 48, 54).

Use of protein microarrays to measure antibody levels has found useful applications in diverse fields during the past 5 years (12, 49). Small sample volumes are required, and many antigens can be screened simultaneously. The technical aspects of probing protein microarrays with antibodies are essentially the same as for conventional ELISAs, and the microarray approach can effectively be considered a microscale fluorescence ELISA. The relationship between anti-VACV IgG levels and neutralizing antibody titers were measured with a cohort of 50 smallpox vaccine recipients and 8 unvaccinated controls. Donors with a wide range of neutralizing antibody titers were selected for examination, since a large range allows for robust correlation analysis. Anti-VACV IgG was measured by endpoint dilution ELISA using VACV lysate as the antigen, and neutralizing antibody titers were measured by determining the PRNT<sub>50</sub> (Fig. 1A). A positive correlation was observed between bulk anti-VACV IgG and neutralizing antibody titers ( $P < 0.0001$ ), with a moderate fit ( $r^2 = 0.56$ ), comparable to what we and others have published for other cohorts of smallpox vaccine recipients (11, 20). Our planned correlation analysis between neutralizing antibody titers and IgG responses to individual VACV surface proteins by microarray ELISA depended on the linearity of the data obtained by microarray ELISA. We validated the microarray ELISA linearity by printing VACV lysate antigen on microarrays and testing the full set of 50 donors and 8 negative controls for anti-VACV IgG levels by both conventional endpoint dilution “macro” ELISA and single-dilution microarray ELISA (Fig. 1B). Excellent correlation was observed between the two data sets ( $P < 0.0001$ ;  $r^2 = 0.78$ ), validating the accuracy of microarray-based IgG mea-

surement and the linearity of the data with the conditions used. The linearity of the microarray results over a large signal range appears to be facilitated both by the use of fluorescently conjugated secondary antibodies and by the high concentration of antigen present in the microarray spots, both preventing saturation and providing a high degree of sensitivity. Anti-VACV IgG as measured by microarray was correlated to neutralizing antibody titers, and a positive correlation was observed ( $P < 0.0001$ ), with a fit comparable to that of the conventional ELISA ( $r^2 = 0.48$ ) (Fig. 1C).

Having validated the microarray technique, we then completed full-scale correlation analysis between each of the 16 VACV MV surface proteins and neutralizing antibody titers of the 50 vaccinated donors. Linear regression analysis was done to determine the presence or absence of correlation between the level of IgG against individual VACV proteins and neutralizing antibody titers. We hypothesized that the presence of an immunodominant neutralizing antibody target could be revealed as the antigen exhibiting the strongest correlation with neutralizing antibody titers. Of the 16 antigens tested, 5 known and 1 putative VACV MV surface proteins exhibited a positive correlation with neutralizing antibody titers (each  $P$  value was  $< 0.05$ ). By this correlation analysis, H3 was the most immunodominant VACV neutralizing antibody target in humans ( $P < 0.0001$ ;  $r^2 = 0.41$ ) (Fig. 2A). A27 ( $P < 0.002$ ;  $r^2 = 0.25$ ), D8 ( $P < 0.0002$ ;  $r^2 = 0.25$ ), A14 ( $P < 0.0002$ ;  $r^2 = 0.19$ ), D13 ( $P < 0.003$ ;  $r^2 = 0.17$ ), and L1 ( $P < 0.02$ ;  $r^2 = 0.12$ ) also exhibited positive correlations with neutralizing antibody titers, though with weaker fits (Fig. 2B to D and F). H3, A27, D8, and L1 have all been previously described as VACV neutralizing antibody targets in mice or rabbits (13, 29, 30, 35, 51), and antibodies against any one of these four targets can protect mice from a lethal VACV challenge (13, 36, 46, 52); therefore, it was perhaps not surprising that these targets were indicated as neutralizing antibody targets in humans by serological correlation analysis. However, neither A14 (Fig. 2D) nor D13 (Fig. 2E) has been previously proposed as a VACV neutralizing antibody target, and they warrant further

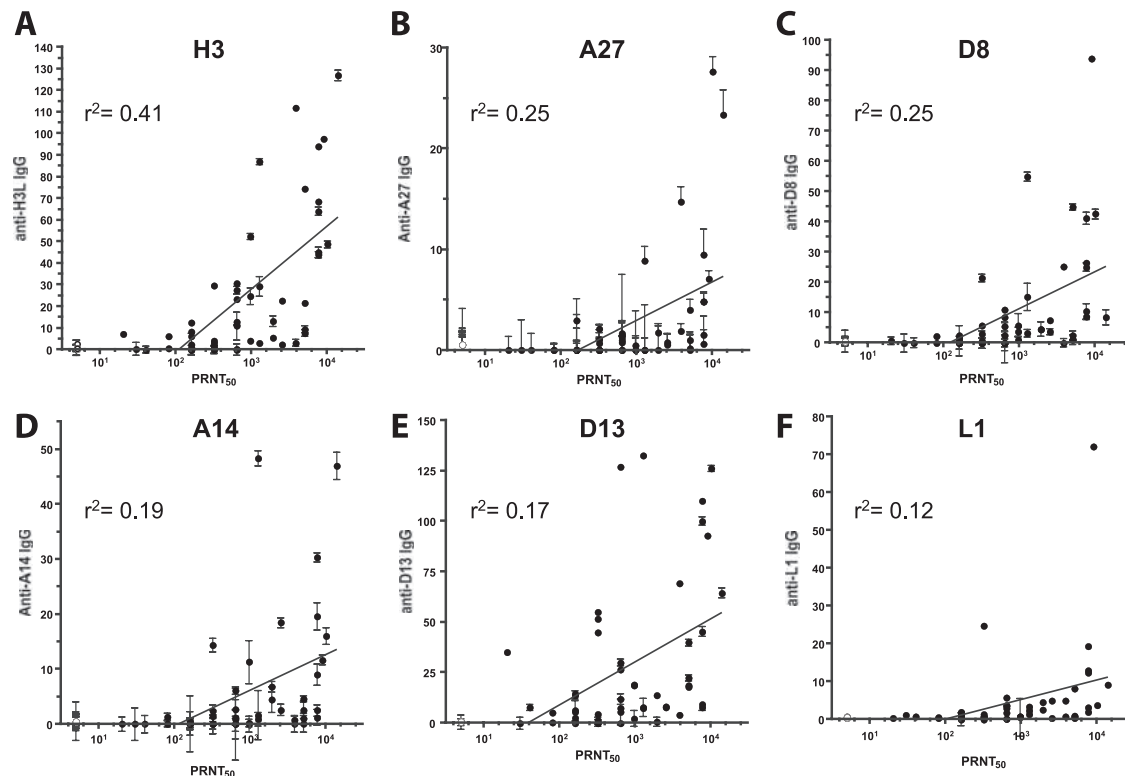


FIG. 2. Correlation of specific antibody response targets with virus neutralization activity. IgG levels specific for individual VACV MV surface proteins were measured by vaccinia viral protein microarray analysis of plasma samples from 50 smallpox vaccine recipients. Separately, VACV neutralizing antibody levels were quantified ( $PRNT_{50}$ ) using the same plasma samples. Linear regression analysis was done to determine the presence or absence of correlation between the levels of IgG (relative units) for each individual VACV protein and neutralizing antibody titers in the cohort of vaccinees. Six known or putative MV surface proteins exhibited a positive correlation with neutralizing antibody titers. Graphs (y axis, anti-VACV protein IgG; x axis,  $PRNT_{50}$ ) are shown in order from highest to lowest correlation ( $r^2$ ): H3,  $r^2 = 0.41$  (A); A27,  $r^2 = 0.25$  (B); D8,  $r^2 = 0.25$  (C); A14,  $r^2 = 0.19$  (D); D13,  $r^2 = 0.17$  (E); L1,  $r^2 = 0.12$  (F). Error bars represent the full range of duplicate samples.

examination. While D13 has clearly been shown to be a scaffolding protein for the morphogenesis of immature particles (10, 39, 57) and is shed from mature particles (MV) (24), data from several labs suggest that a fraction of D13 molecules remain associated with mature intracellular virions (9, 48) and therefore may serve as a surface target for antibody responses. It was also interesting that no antibodies to any of the other known MV surface proteins (e.g., A21, A17, A9, F9, and H2) correlated with virus neutralization activity, suggesting either that only some surface antigens can be neutralization targets or that those antigens that do not correlate fail to correlate because they are insufficiently immunogenic, resulting in unmeasurable IgG levels for most vaccinees (Table 1).

While H3 was the most immunodominant VACV neutralizing antibody target in humans by correlation analysis, the  $r^2$  value (0.41) suggested that other factors beyond anti-H3 IgG levels are important for determining neutralizing antibody titers. The simplest interpretation was that two major neutralizing antibody targets exist in vaccinated humans, H3 and one other. Obvious candidates were the five other proteins exhibiting a positive correlation with neutralization. To test this hypothesis, we examined various combinations of antibodies (e.g., the sum of the anti-H3 and anti-A27, anti-H3 and anti-L1, or anti-A27 and anti-L1 IgG signals) for a high degree of correlation ( $r^2 \geq 0.6$ ) with neutralizing antibody titers. In no

case did a combination of two (or even three) potential neutralizing antibody targets reveal a significant improvement in correlation over H3 alone (data not shown).

**H3 and L1 immunodominance.** Anti-H3 IgG was detected in most vaccinees (Fig. 2A and 3A; Table 1), and the levels of anti-H3 IgG correlated both with the magnitude of the neutralizing antibody response (Fig. 2A) and with that of the overall anti-VACV IgG response ( $P < 0.0001$ ;  $r^2 = 0.47$ ) (Fig. 3A). Strong anti-L1 antibody responses have been reported for VACV-infected mice and have been the subject of much investigation (26, 31, 36, 56, 61). Therefore, we were surprised to find that humans make a poor antibody response to L1 (Fig. 3B). While 40% of vaccinees make an anti-L1 response above the limit of detection (Table 1), it was striking that few vaccinees make a strong antibody response to L1. Only 10% of vaccinees make an IgG response to L1 that is >10-fold above the limit of detection (Table 1). This is shown graphically in Fig. 3B, where only a few donors make notable anti-L1 IgG responses. The L1 protein used in this study is appropriately folded, and the protein can be crystallized (56). Nevertheless, to further determine whether the low anti-L1 IgG levels were due to aspects of the experimental techniques, we tested serum samples from mice infected with VACV for anti-L1 IgG levels (Fig. 3C to E). While most human donors exhibited a strong anti-H3 IgG response and a weak or undetectable anti-L1 IgG

TABLE 1. Specific IgG responses in serum samples from vaccinees

Description	VACV protein	% Seropositive ( <i>n</i> <sup>a</sup> )		% Vaccinated donors whose IgG levels were >10× LOD <sup>b</sup>
		Vaccinated donors	Unvaccinated donors	
MV membrane proteins	A9	0 (0)	0 (0)	0
	A13	10 (5)	0 (0)	0
	A14	36 (18)	0 (0)	16
	A17	2 (1)	0 (0)	0
	A27	24 (12)	0 (0)	6
	D8	54 (27)	0 (0)	18
	D13	76 (38)	0 (0)	50
	E10	4 (2)	0 (0)	2
	H2	4 (2)	0 (0)	0
	H3	74 (37)	0 (0)	44
	I5	10 (5)	0 (0)	2
	L5	12 (6)	0 (0)	2
	L1	40 (20)	0 (0)	10
	F9	2 (1)	0 (0)	0
	A21	0 (0)	0 (0)	0
	A28	0 (0)	0 (0)	0
Core protein	I1	76 (38)		46
Replication protein	A26	86 (43)		58
Negative controls	IVT1	2 (1)	0 (0)	0
	IVT2	2 (1)	0 (0)	0

<sup>a</sup> *n*, no. of seropositive donors.

<sup>b</sup> LOD, limit of detection.

response (Fig. 3C and D), mice exhibited strong antibody responses to both H3 and L1. These results were further quantified by normalizing the anti-H3 and anti-L1 antibody responses to the total VACV response (VACV lysate) in both humans and mice (Fig. 3E). The normalized data show that H3 and L1 are codominant in mice (with responses to L1 being somewhat higher than those to H3), while H3 is greatly immunodominant to L1 in vaccinated humans (Fig. 3E). Therefore, while L1 is a strong neutralizing antibody target in mice, it appears to be a minor component of the human antibody response. These results illustrate a clear species difference in the antibody responses to VACV.

**Identifying immunodominant surface antibody targets other than H3.** While anti-H3 IgG was best correlated with neutralizing antibody titers in vaccinees, the notion that anti-H3 IgG is not the only major VACV neutralizing antibody target was reinforced by more-detailed examination of the antibody response profiles of vaccinees. Seventy-four percent of vaccinees were seropositive for anti-H3 IgG, while 100% of vaccinees were positive for VACV neutralizing antibodies (Table 1; Fig. 2). This indicated that at least some donors likely make use of an alternative neutralizing antibody target. A case-by-case approach was then taken: for a given vaccinee, if anti-H3 antibodies are not present, what potential neutralizing responses are present? IgG responses to VACV MV surface antigens could be represented as a panel of 16 bar graphs for each individual vaccinee (Fig. 4). A signal level of 2 IgG RU was selected as a stringent cutoff (Fig. 4), establishing 98% specificity overall (see Table 1, negative controls). Three representative vaccinees are shown in Fig. 4A to C, along with one

unvaccinated control (Fig. 4D). It was possible to identify vaccinees with strong neutralizing antibody titers and who exhibited an immunodominant anti-H3 response (Fig. 5A). Donors that likely depended predominantly on neutralizing antibodies to targets other than H3 could be identified by selecting individuals with no or low anti-H3 IgG but who nevertheless possessed significant neutralizing antibody titers, revealed by plotting anti-H3 IgG versus the PRNT<sub>50</sub> (Fig. 5B). Other candidate neutralizing antibody targets could then be assessed by examining the antibody responses to the panel of remaining known MV surface proteins (bar graphs as per Fig. 4). By such analysis, a vaccinee was identified with an immunodominant anti-L1 IgG response (71% of measurable anti-MV surface protein IgG) (Fig. 5B, upper graph), implicating L1 as a major neutralizing antibody target in this particular individual. In contrast, a representative vaccinee with the same neutralizing antibody titer exhibited no detectable anti-L1 IgG ( $\leq 2.7\%$  of measurable anti-MV surface protein IgG) (Fig. 5B, lower

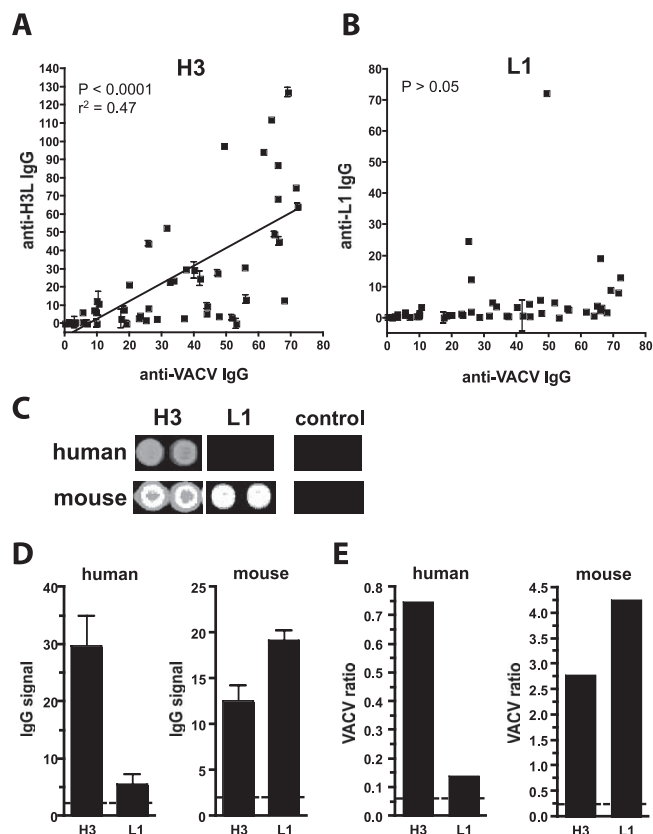


FIG. 3. Immunodominance comparison of H3 and L1 antigens in humans and mice. (A) Anti-H3 IgG graphed against total anti-VACV IgG (VACV lysate antigen) for each of the 50 donors (individual squares).  $P < 0.0001$ ;  $r^2 = 0.47$ . Error bars represent the full range of duplicate samples. (B) Anti-L1 IgG graphed against total anti-VACV IgG (VACV lysate antigen) for each of the 50 donors ( $P > 0.05$ ). (C) Example microarray spots of H3 and L1 proteins probed with human and mouse plasma and serum samples. (D) Average IgG signals to H3 and L1 in human or mouse samples. An IgG signal level of 2 RU (relative units) was selected as a stringent cutoff (dashed line), establishing 98% specificity (Table 1 and Materials and Methods). (E) Ratio of anti-H3 IgG to VACV lysate IgG or anti-L1 IgG to VACV lysate IgG for vaccinated humans (left) or mice (right).

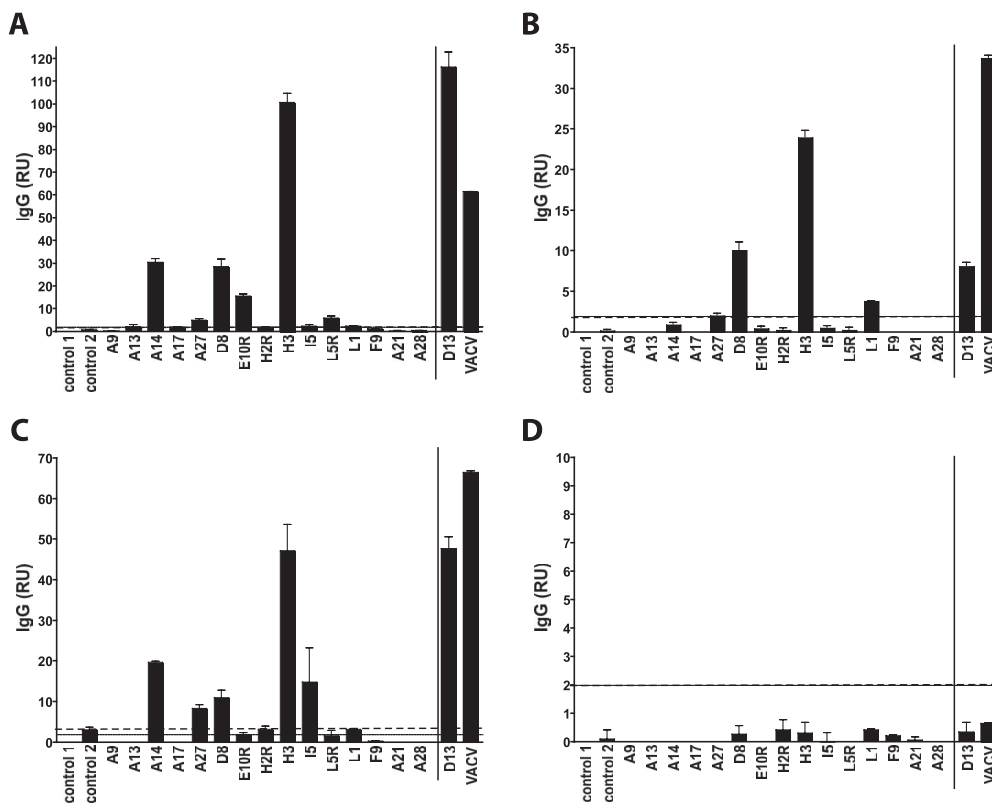


FIG. 4. Antibody profiles of individual vaccinees. IgG responses to known and potential VACV MV surface antigens are shown for three human vaccinees (A to C) and one unvaccinated donor (D). Two negative control antigens are shown on the left side of each graph. VACV lysate was used as a positive control and is shown to the right of the dividing line, along with D13, which has uncertain surface expression. Dashed line, limit of detection. Error bars represent the full range of duplicate samples.

graph), indicating that anti-L1 IgG is not necessary for virus neutralization activity in human vaccinees. Instead, strong responses to H3, D8, and A14 were measured (Fig. 5B, lower graph).

By continuing the case-by-case approach for identifying other candidate neutralizing antibody targets in humans, a vaccinee was identified with a minimal anti-H3 IgG response and an immunodominant anti-A27 IgG response (60% of measurable anti-MV surface protein IgG [see Fig. S2A, upper graph, in the supplemental material]), implicating A27 as a major neutralizing antibody target in this particular individual. In contrast, a representative vaccinee with the same neutralizing antibody titer exhibited no detectable anti-A27 IgG ( $\leq 1.3\%$  of measurable anti-MV surface protein IgG [see Fig. S2A, lower graph, in the supplemental material]), indicating that anti-A27 IgG is not necessary in vaccinees for virus neutralization activity.

A different vaccinee was identified with a minimal anti-H3 IgG response and an immunodominant anti-A14 IgG response (63% of measurable anti-MV surface protein IgG [see Fig. S2B, upper graph, in the supplemental material]). In contrast, a representative vaccinee with a similar neutralizing antibody titer exhibited no detectable anti-A14 IgG ( $\leq 3\%$  of measurable anti-MV surface protein IgG [see Fig. S2B, lower graph, in the supplemental material]), indicating that anti-A14 IgG is not necessary in vaccinees for virus neutralization activity.

Overall, the diversity and variability of the antibody re-

sponses we observed in different vaccinees were unexpected and impressive. We observed no stereotypic antibody signature in vaccinated people (Fig. 3 to 5; Table 1) (see Fig. S2 in the supplemental material). While anti-H3 antibodies were implicated as the immunodominant neutralizing antibody target in most vaccinees, we also observed statistical evidence for neutralizing antibody responses to A27, D8, A14, D13, and L1, at least in subsets of vaccinees.

**Blockade of anti-H3 virus neutralizing antibodies.** Correlation does not equal causation. Therefore, while anti-H3 is the immunodominant surface antigen target in a majority of vaccinees as determined by microarray ELISA and anti-H3 IgG exhibited the best correlation with the neutralizing antibody titer, to test the prediction that anti-H3 IgG is the predominant VACV neutralizing antibody specificity in vaccinated humans it was necessary to remove the anti-H3 IgG and measure the remaining VACV neutralization activity. The simplest approach to eliminate anti-H3 IgG activity was to preincubate plasma samples with an excess of rH3, such that all available anti-H3 IgG was bound to rH3, and then test the VACV neutralization activity of the anti-H3-blocked plasma sample. The prebound anti-H3 IgG would be expected to then be unavailable to neutralize VACV. Plasma from a vaccinee was incubated with 10  $\mu\text{g}$  rH3 and then screened by VACV microarray ELISA to determine the completeness of anti-H3 adsorption. H3 binding was completely blocked (99.97% [Fig. 6A]), but D8 binding was unaffected (Fig. 6B),

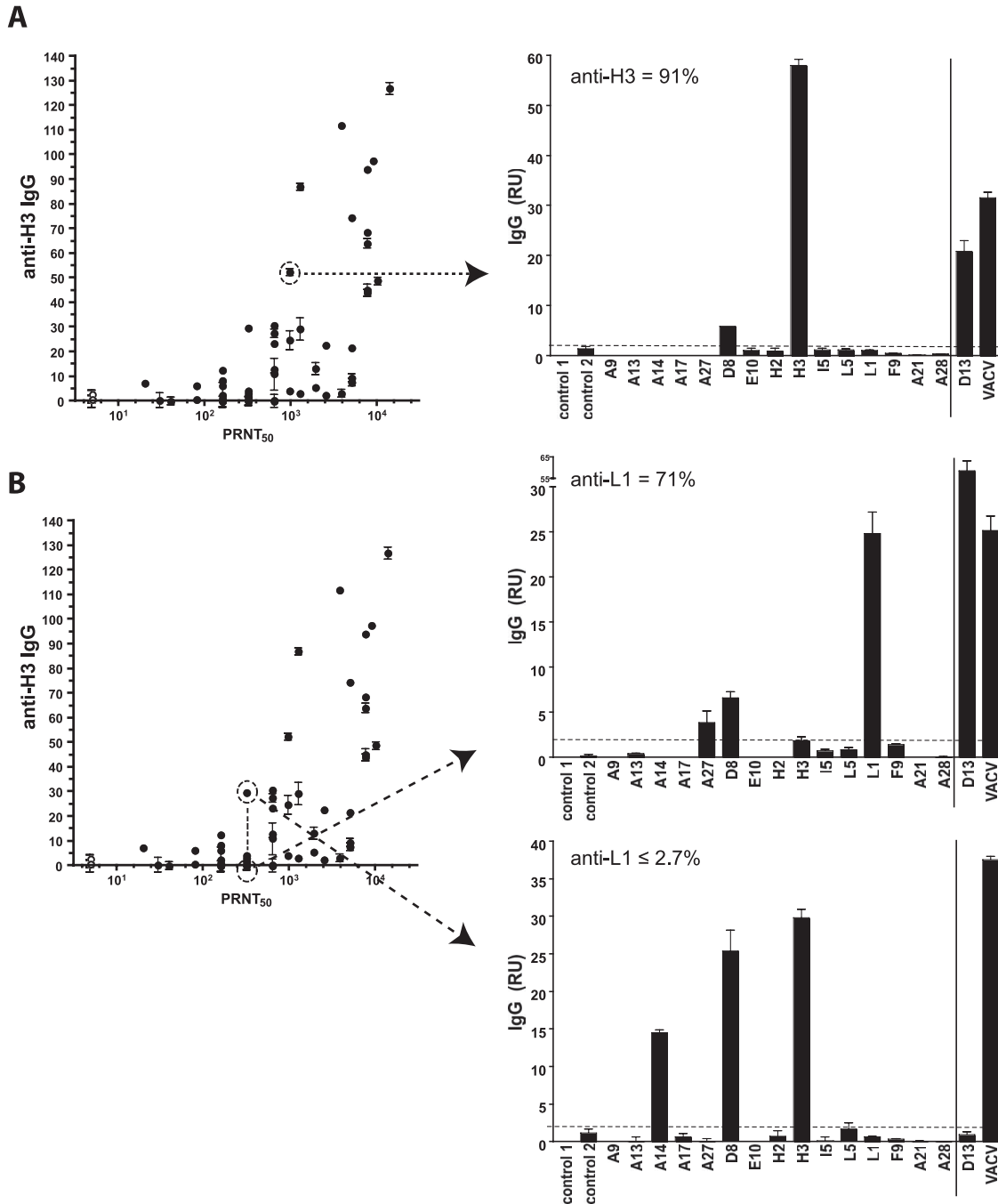


FIG. 5. Variable anti-H3 and anti-L1 IgG responses in vaccinated humans. (A) Human vaccinee identified with a highly immunodominant IgG response to H3 among the MV surface proteins (91% of measurable anti-MV surface protein IgG). (B) Donors that likely depend on neutralizing antibodies to targets other than H3 could be identified by selecting individuals with no or low anti-H3 IgG but who nevertheless possessed significant neutralizing antibody titers (anti-H3 versus PRNT<sub>50</sub>). Other candidate neutralizing antibody targets could be assessed by examining the antibody responses to the remaining known MV surface proteins. By such analysis, a vaccinee was identified with an immunodominant anti-L1 IgG response (upper graph; anti-L1 highlighted in black). In contrast, a representative vaccinee with the same neutralizing antibody titer exhibited no detectable anti-L1 IgG (lower graph,  $\leq 2.7\%$  of measurable anti-MV surface protein IgG), indicating that anti-L1 IgG is not necessary for virus neutralization activity. Dashed lines, limits of detection.

demonstrating completeness of the blockade and the specificity of the blockade. Other specificities were also unaffected (Fig. 6C).

VACV neutralization activity of the anti-H3 blocked plasma was then tested. Surprisingly, no decrease in neutralization activity was observed (Fig. 6D). A titration of rH3 doses used

to adsorb the anti-H3 IgG was done, confirming that 10  $\mu\text{g}$  was well above the level needed to saturate all available anti-H3 antibody (Fig. 6E), and increasing the amount of rH3 in the blockade still had no appreciable effect on viral neutralization activity (Fig. 6F). Free rH3 was able to block the anti-H3 IgG

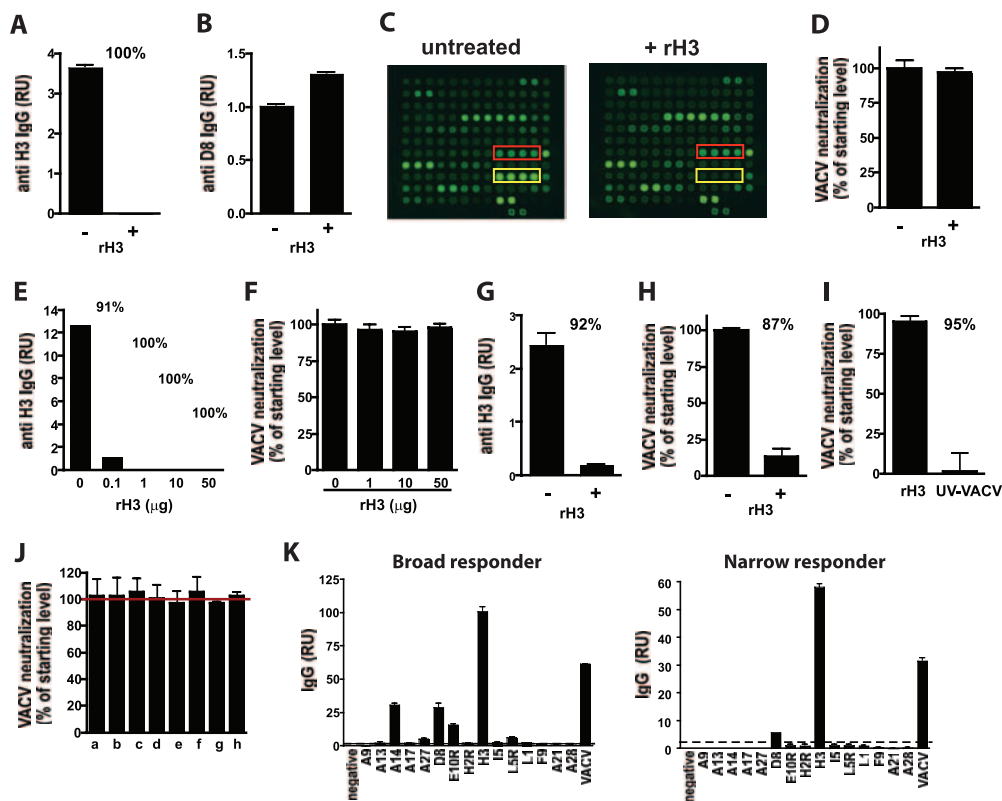


FIG. 6. Effects of blockade of anti-H3 antibodies on virus neutralization activity of human plasma. (A to D) Plasma from a human smallpox vaccinee was blocked with the rH3 protein and tested for efficiency of the blockade, specificity of the blockade, and the effect of the blockade on VACV neutralization activity. Quantitation of anti-H3 (A) or anti-D8 (B) binding IgG in plasma after anti-H3 antibodies were blocked with rH3 (10 μg) (+) or left untreated (-). (C) Raw data from the VACV viral protein microarrays. H3 protein spots (yellow box) and D8 protein spots (red box) in untreated plasma (plasma alone) or blocked plasma (+ rH3). (D) VACV neutralizing antibody levels with or without rH3 blockade (percentage of starting level quantified). Data are representative of numerous experiments. (E) Quantitation of anti-H3 IgG binding in plasma from a vaccinee blocked with different amounts of rH3 protein (0, 0.1, 1, 10, or 50 μg). (F) Quantitation of VACV neutralizing antibody activity in the plasma samples from panel E. Data in (E and F) are representative of two experiments. (G and H) Serum from an H3 protein-immunized rabbit was incubated with 10 μg of rH3 protein (+) to block anti-H3 IgG activity and then tested for anti-H3 binding IgG (reduced 92% by blockade) (G) or VACV neutralizing antibody activity (reduced 87% by blockade) (H). (I) VACV neutralizing antibody activity measured after preincubation with 10 μg of rH3 protein or UV-VACV (10<sup>7</sup> PFU equivalent). Pooled data of samples from seven human donors are shown. (J) Percentage of VACV neutralizing antibody activity in plasma from each human donor (a to h) measured after preblocking with the rH3 protein (data using 1- or 10-μg treatment were pooled since no difference was observed between the two groups). The Error bar indicates the full data range. The red line indicates 100% of starting VACV neutralization activity in untreated plasma from each individual vaccinee. (K) Quantitation of anti-VACV-specific IgG signal intensities from VACV protein microarray ELISA probed with sera from two different smallpox vaccinees used for this figure. The error bar indicates the full data range. Dashed line, limit of detection. Error bars in panels show SEM unless otherwise indicated. Data are representative of multiple experiments.

(92%) and VACV neutralization activity (87%) of serum from H3-immunized rabbits (Fig. 6G to H;  $P < 0.001$  and  $P < 0.005$ ), demonstrating that rH3 was capable of binding anti-H3 neutralizing antibodies and able to functionally block those antibodies. We confirmed that it was possible to fully block the VACV neutralizing antibodies (95%) by preincubation of human plasma samples with UV-inactivated VACV ( $P < 0.0001$ ) (Fig. 6I). We then tested a panel of eight different human vaccinees to exclude the possibility that the first vaccinee was in some way unusual. All eight donors, selected from a wide range of time points postvaccination, gave identical results: blockade of anti-H3 IgG resulted in no appreciable reduction in VACV neutralization activity (<5% change [Fig. 6J]). This was observed whether the donor exhibited broad or narrow anti-VACV surface protein IgG responses (Fig. 6K). These results were quite surprising. Upon final analysis, we did not trust the

free protein blockade experiments because of general concerns about the addition of a large molar excess of viral receptor (H3) to the reactions and the fact that the anti-H3 antibodies were still physically present throughout the experiments.

**Depletion of anti-H3 virus neutralizing antibodies: reverse immunoprecipitation.** Given the concerns about the blockade approach, we developed an experimental approach based on the physical removal of anti-H3 IgG from vaccinee plasma samples using a reverse immunoprecipitation strategy. His-tagged rH3 was bound to Ni-Sepharose, providing a solid matrix to which anti-H3 IgG could be adsorbed from human plasma samples. The anti-H3 IgG bound to the rH3-Ni-Sepharose was then physically separated from the plasma by a simple centrifugation. The anti-H3 IgG was pelleted as part of the Ni-Sepharose, leaving the anti-H3-depleted plasma (Fig. 7A). Plasma from a vaccinated individual was treated with rH3-Ni-

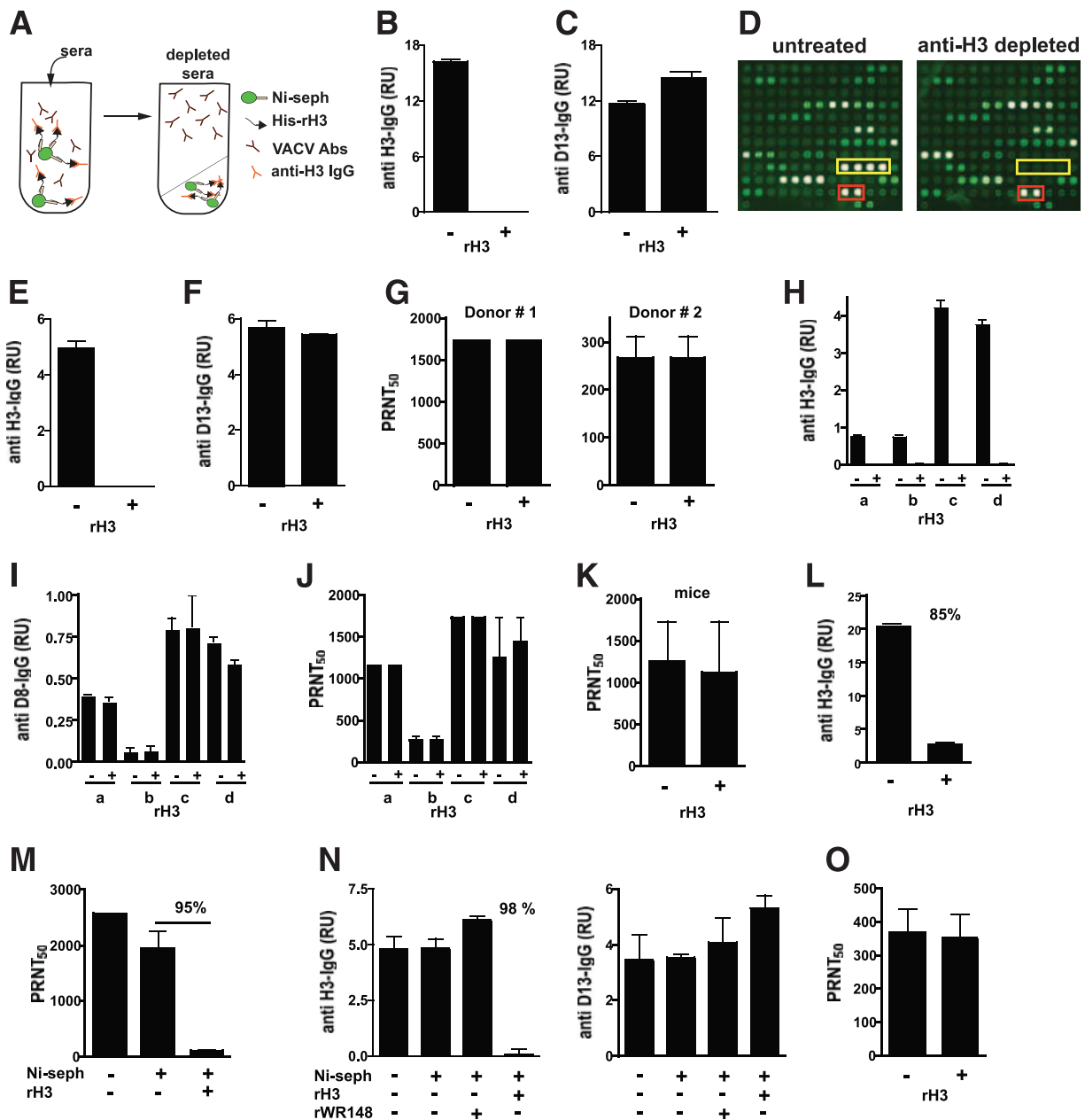


FIG. 7. Reverse immunoprecipitation: effects of depletion of anti-H3 antibodies on virus neutralization activity of human plasma. (A) H3-specific antibodies were depleted by conjugation of His-rH3 to Ni-Sepharose agarose ("Ni-seph"), incubation with plasma from a vaccinee, and centrifugation. Quantification of anti-H3 (B and E) or anti-D13 (C and F) IgG signal intensities from donor #1 (B and C) or donor #2 (E and F), untreated (-) or after anti-H3 antibody depletion (+). (D) Representative array scan of serum from donor #1 showing spots of H3 (yellow box) or the control (VACV lysate, red box) in untreated serum (whole plasma) or anti-H3 antibody-depleted serum with 10  $\mu$ g of rH3 (H3-depleted plasma). (G) VACV neutralizing antibody titers (PRNT<sub>50</sub>) in smallpox-vaccinated donors #1 and 2 measured before (-) and after (+) anti-H3 antibody depletion. Data are representative of multiple experiments. (H to J) Depletion of H3-specific antibodies in samples from four total vaccinees (a to d). Quantification of anti-H3 (H) or anti-D8 (I) IgG in each human donor (a to d) measured before (-) and after (+) anti-H3 antibody depletion. (J) VACV neutralizing antibody titers (PRNT<sub>50</sub>) in each human donor (a to d) measured before (-) and after (+) anti-H3 antibody depletion. (K) VACV neutralizing antibody titers (PRNT<sub>50</sub>) in VACV-infected mice measured before (-) and after (+) anti-H3 antibody depletion. Quantification of anti-H3 (L) or VACV neutralizing (M) antibody titers (PRNT<sub>50</sub>) in H3 protein-immunized rabbits measured before (-) and after (+) anti-H3 antibody depletion. Ni-Sepharose-alone control is shown. (N) Quantitation of anti-H3 and anti-D13 IgG when the order of the anti-H3 IgG depletion was reversed. Plasma was preincubated with His-tagged rH3 and then complexed with Ni-Sepharose. Samples were untreated, treated with Ni-Sepharose alone, or depleted using one of two His-tagged VACV proteins: rH3 or WR148. (O) VACV neutralizing antibody titers (PRNT<sub>50</sub>) measured in plasma samples from panel N, pretreated as indicated. All data are representative of multiple experiments. Error bars indicate SEM in each condition, except in panel I, where error bars represent the full data range of replicate samples.

Sepharose to deplete anti-H3 IgG and then tested by VACV microarray ELISA as a convenient and robust strategy to validate the specificity and completeness of the depletion (Fig. 7D). Anti-H3 IgG was completely eliminated (100%) (Fig. 7B), but anti-D13 was unaffected (Fig. 7C). Anti-H3 IgG was depleted from the plasma of a second vaccinee in the same manner, with comparable efficacy (Fig. 7E and F).

VACV neutralization activity of the anti-H3-depleted plasma was then tested. No decrease in VACV neutralization activity was observed for either donor (Fig. 7G). A total of four vaccinees were tested, with identical results (Fig. 7H to J). This was not a result restricted to human vaccinees, since identical results were obtained after depletion of anti-H3 IgG from serum from VACV-infected mice (Fig. 7K). It was conceivable that there were two types of anti-H3 IgG present in the plasma of human vaccinees, neutralizing and nonneutralizing, and the rH3 was capable of binding only the nonneutralizing anti-H3 IgG. To confirm that all anti-H3 IgG was bound and depleted, we utilized serum from rabbits containing high levels of anti-H3 neutralizing IgG. The anti-H3 was depleted (85%), and viral neutralization activity was abolished (95% reduction) (Fig. 7L and M), demonstrating that rH3 binds neutralizing anti-H3 IgG. In addition, we have generated panels of anti-H3 neutralizing antibodies, and the rH3 protein efficiently binds those anti-H3 neutralizing monoclonal antibodies (S. Crotty and colleagues, unpublished data).

It was possible that prebinding rH3 to the Ni-Sepharose matrix shielded the dominant neutralizing epitope. To control for this possibility, the binding order was reversed. Plasma samples were preincubated with His-tagged rH3 and then incubated with Ni-Sepharose to adsorb the anti-H3 IgG-rH3 complexes, which were removed by centrifugation as described above. Reversal of the binding order had no significant effect, since anti-H3 IgG was still fully depleted (98%) (Fig. 7N) but VACV neutralization activity was unaffected (Fig. 7O).

**Anti-H3 IgG affinity purification.** The combined results of the H3 blockade experiments (Fig. 6) and H3 reverse immunoprecipitation depletion experiments (Fig. 7) suggested that anti-H3 antibodies are not a major component of VACV neutralizing antibody activity in vaccinated humans, even though the presence of anti-H3 IgG is the best correlated neutralizing antibody target (Fig. 2A). Furthermore, in previous work we and others have shown that H3 is a target of neutralizing antibodies in H3 protein-immunized rabbits (13, 35) and mice (13) and that anti-H3 neutralizing antibodies can protect mice from a lethal poxvirus infection (13). In an earlier study, we showed that we were able to purify anti-H3 IgG from VIG, a commercial product consisting of purified and concentrated IgG from plasma of a cohort of smallpox-vaccinated donors (13). While H3 is an immunodominant target of the human antibody response to the smallpox vaccine (Table 1) and can be the target of human neutralizing antibodies (13), our new results raised the possibility that the anti-H3 antibodies present in most vaccinees are nonneutralizing. These were apparently conflicting results, with some experiments indicating that H3 was a major neutralizing antibody target and other experiments indicating that H3 is not a neutralizing antibody target.

To resolve whether or not H3 is a neutralizing antibody target in smallpox-vaccinated humans, it would be necessary to isolate anti-H3 IgG from vaccinated individuals and directly

test the neutralization activity of the anti-H3 antibodies. While the reverse immunoprecipitation strategy employed above was effective at depleting anti-H3 IgG from human plasma samples (Fig. 7), we were unable to recover purified anti-H3 IgG from the samples, since the rH3 affinity for the anti-H3 Ig was substantially higher than the affinity of the histidine tag for the Ni matrix (data not shown). We therefore developed an affinity purification column chromatography strategy based on covalent linkage of rH3 to an *N*-hydroxy-succinimide column matrix to isolate purified human anti-H3 IgG from vaccinated donors (Fig. 8A). Plasma from individual vaccinated donors was passed over the rH3 column (see Methods). Flowthrough fractions were completely depleted of anti-H3 IgG (Fig. 8B to D), and the depletion was specific, since other anti-VACV IgG specificities were not depleted (Fig. 8C). The column was washed extensively to remove nonspecifically bound proteins (Fig. 8B and C), and then anti-H3 IgG bound to the column was eluted using a low-pH buffer (Fig. 8B, C, and E). The eluted protein was pure anti-H3 IgG, since no other specificities were detected by VACV microarray ELISA (Fig. 8C).

VACV neutralization activity of the purified human anti-H3 IgG was tested by standard viral neutralization assay, using a column wash fraction as a negative control. Purified human anti-H3 IgG exhibited clear VACV neutralization activity (Fig. 8F). To determine the potency of the anti-H3 IgG, the IgG concentration of the purified protein was determined by human IgG ELISA (48  $\mu\text{g/ml}$ ) (Fig. 8G) and the potency calculated as VACV neutralization-specific activity:  $\mu\text{g/ml}$  IgG necessary to neutralize 50% of VACV PFU (Fig. 8H; reciprocal value shown for ease of visualization). Purified human anti-H3 IgG neutralized 50% VACV PFU at 0.5  $\mu\text{g/ml}$ , which was a fourfold increase in specific activity over that of whole plasma from the vaccinee (Fig. 8H). This result demonstrated that anti-H3 antibodies in this smallpox vaccine-immunized donor were indeed neutralizing, with substantial potency (Fig. 8F and H).

Knowing that the anti-H3 antibodies possessed virus neutralization activity and knowing that we had depleted all anti-H3 IgG from the vaccinated donor plasma (Fig. 8B to E), we could definitively address the question of what percentage of antiviral neutralization activity is due to anti-H3 IgG. VACV neutralization assays were performed on H3-depleted (flowthrough) and undepleted (whole plasma) plasma samples. VACV neutralization by the two samples was indistinguishable (Fig. 8I). This result is discussed further below (see Discussion).

Given the variability observed between human vaccinees (Fig. 3 to 5) (see Fig. S2 in the supplemental material), we repeated the full set of anti-H3 IgG purification experiments using plasma from a second donor ("donor #2") (Fig. 8J to O). One hundred percent of anti-H3 IgG was depleted from the plasma (Fig. 8J and K), and pure anti-H3 IgG was eluted from the column (Fig. 8J and L). Anti-H3 IgG from donor #2 exhibited neutralization activity (Fig. 8M), and that neutralization activity possessed a specific activity very similar to that of the anti-H3 obtained from donor #1 (0.625 and 0.5  $\mu\text{g/ml}$  IgG<sup>-1</sup>, respectively) (Fig. 8H and 8N). Anti-H3 IgG consisted of a larger fraction of the neutralizing antibodies in donor #2 than donor #1, since the purified anti-H3 from donor #2 represented a 16-fold increase in specific activity over that for whole plasma (Fig. 8N). Nevertheless, when depleted and undepleted plasma samples from donor #2 were compared for

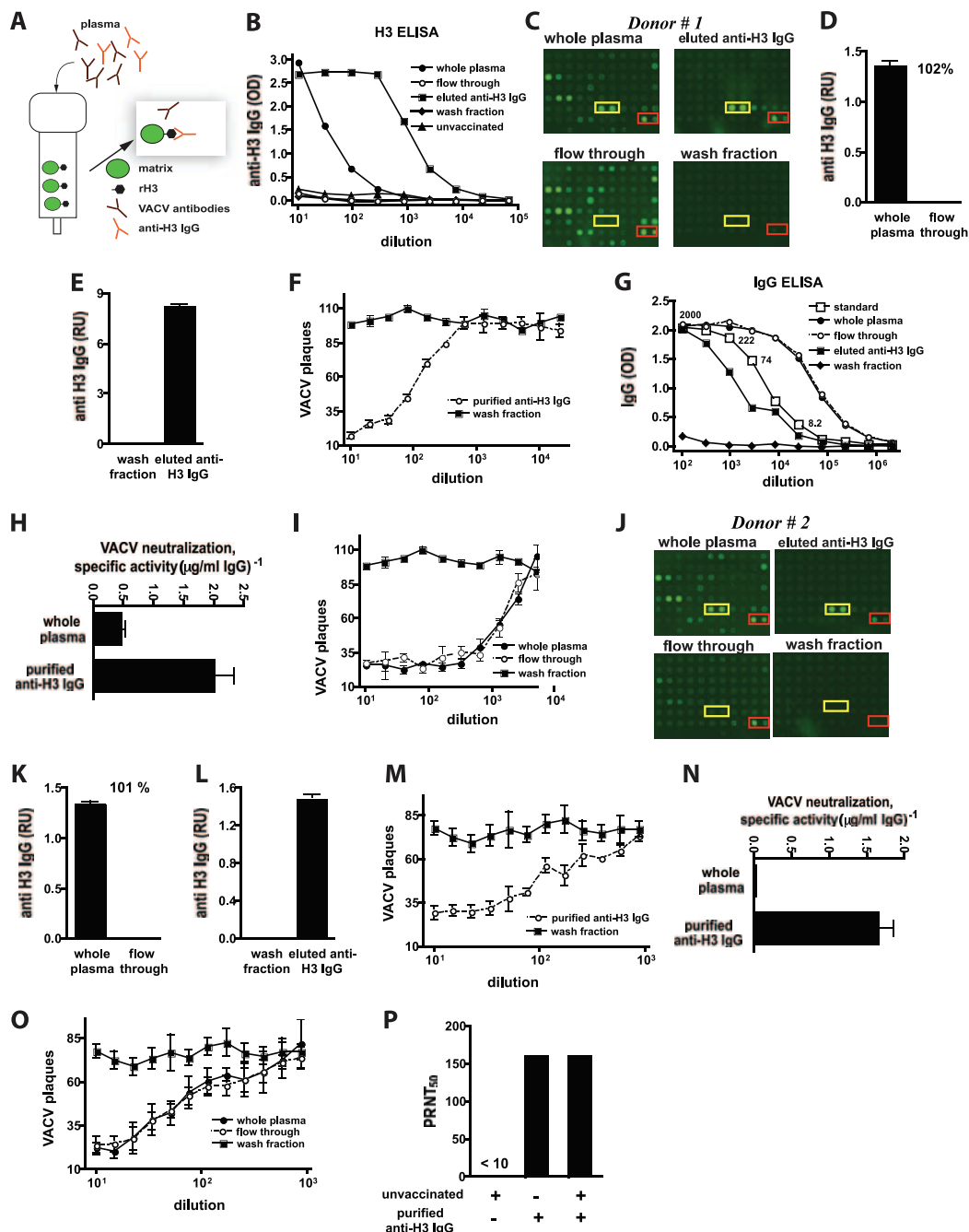


FIG. 8. Affinity purification of human anti-H3 antibodies from smallpox vaccinees. Antiviral antibodies from human smallpox vaccinee donor #1 (B to I) and donor #2 (J to P) were examined after depletion or purification of anti-H3 Ig using affinity column chromatography. (A) Anti-H3 IgG was purified from human plasma by rH3 affinity chromatography. Column flowthrough fractions were collected, as were wash fractions. Anti-H3 IgG was eluted by a low-pH wash ("eluted anti-H3 IgG"). (B) Anti-H3 IgG in fractions was quantified by H3 ELISA. Plasma from an unvaccinated donor was used as a negative control. (C) Raw microarray data; H3 protein (yellow box) and control (total VACV lysate, red box). (D) Quantitation of depletion of anti-H3 IgG from the plasma sample, determined by VACV protein microarray ELISA. (E) Quantitation of purification of anti-H3 IgG, as per panel D. (F) VACV neutralizing antibody activity of purified human anti-H3 IgG from donor #1, determined by serial dilution. Plaque assay results are graphed. The affinity column wash fraction was used as a negative control. (G) Absolute concentrations of IgG in samples were determined by ELISA, using an IgG standard (open squares). (H) VACV neutralization specific activity ( $\mu\text{g/ml IgG}^{-1}$ ) calculated for whole plasma and purified anti-H3 IgG from smallpox vaccinated donor #1. (I) VACV neutralizing antibody activity of anti-H3-depleted plasma ("flow through") and untreated plasma ("whole plasma") samples from donor #1, determined by serial dilution. Plaque assay results are graphed. The column wash fraction was used as a negative control (experiment done concurrently with that shown in panel F). (J) VACV protein microarray ELISA scan of affinity column samples from donor #2. Spots of the H3 protein (yellow box) and control (total VACV lysate, red box) are highlighted. Anti-H3 IgG levels quantified in whole plasma and flowthrough fractions (K) or wash fractions (L) versus eluted anti-H3 IgG. (M) VACV neutralizing antibody activity. (N) Specific activity of purified human anti-H3 IgG from donor #2. (O) VACV neutralizing antibody activity of anti-H3-depleted plasma ("flow through") and untreated plasma ("whole plasma") samples from donor #2, determined by serial dilution. Plaque assay results are graphed. (P) VACV neutralization activity (PRNT<sub>50</sub>) determined for purified anti-H3 IgG, plasma from an unvaccinated donor, and a mixture of the two. All data are representative of multiple experiments. Error bars indicate SEM in each condition.

virus neutralization activity, anti-H3-depleted and undepleted samples were indistinguishable (Fig. 8O).

These results were perplexing. One possible explanation was that nonspecific factors in plasma combine with the antigen-specific antibodies to greatly potentiate their activity, such that comparison of purified Ig with Igs in plasma was grossly inaccurate. This possibility was tested by spiking plasma from an unvaccinated donor, possessing no VACV neutralization activity, with purified anti-H3 IgG (Fig. 8P). Purified human anti-H3 IgG exhibited the same PRNT<sub>50</sub> value in the presence or absence of plasma (Fig. 8P).

**L1 as a potential human neutralizing antibody target: examination by blockade or depletion.** Would another potential neutralizing antibody target give results similar to those with H3? L1 is a known neutralizing antibody target in mice, and a small subset of human vaccinees make strong antibody responses to L1 (Fig. 3, 5, and 9A). This led us to examine whether human anti-L1 antibody responses are neutralizing and whether those neutralizing antibodies can be immunodominant contributors to neutralization in vaccinees with strong anti-L1 responses. The same experimental approaches were used to examine the role of human anti-L1 antibody responses to the smallpox vaccine (Fig. 9 and 10) as were used to study anti-H3 antibodies (Fig. 6 to 8).

As a first approach, human plasma samples known to contain anti-L1 activity were preincubated with an excess of rL1, such that all available anti-L1 IgG was bound to rL1 and thereby putatively sequestered (Fig. 9B and C). L1 binding was completely blocked (99%) (Fig. 9B), but anti-D8 was unaffected (Fig. 9B). No decrease in VACV neutralization activity was observed (Fig. 9D). Five additional anti-L1-seropositive smallpox vaccine-immunized donors were tested in the same manner, with the same results (Fig. 9E and F). A titration of rL1 doses used to adsorb the anti-L1 IgG was done, confirming that 10 µg was well above the level needed to saturate all available anti-L1 antibody (data not shown), and increasing the amount of rL1 in the blockade still had no appreciable effect on viral neutralization activity (Fig. 9G). Given that L1 is known to be a neutralizing antibody target in VACV-immunized mice, we tested the effect of blocking anti-L1 antibodies in serum from VACV-immunized mice. Blockade of murine anti-L1 antibodies had no effect on VACV neutralizing antibody activity (Fig. 9H). As a final test of this experimental approach, neutralizing anti-L1 serum from rabbits immunized with L1 was blocked with rL1 *in vitro*. Anti-L1 Ig binding was saturated (measured by protein microarray; data not shown), and VACV neutralization activity was fully blocked (data not shown), demonstrating that neutralizing anti-L1 antibodies are blocked by this technique.

As a second approach, the His-tagged rL1 was bound to Ni-Sepharose and used to reverse immunoprecipitate anti-L1 IgG from human plasma samples known to be seropositive for L1. Anti-L1 IgG was fully depleted from the samples (Fig. 9I; also data not shown), yet the depletion of anti-L1 had no effect on VACV neutralizing antibody activity (Fig. 9J; also data not shown). Reversing the binding order for the depletion (rL1 plus plasma plus Ni-Sepharose) also resulted in effective depletion of human anti-L1 IgG (Fig. 9K) but still had no significant effect on the VACV neutralization activity of the plasma samples (Fig. 9L).

It was plausible that anti-L1 and anti-H3 antibodies may com-

pensate for each other when one is blocked or depleted. Therefore, we blocked both anti-H3 and anti-L1 IgGs simultaneously from nine vaccinees who were determined to be seropositive for both antigens. No reduction in VACV neutralizing antibody activity was observed (Fig. 9M). In addition, we knew that mice infected with VACV made strong responses to both H3 and L1 (Fig. 3); therefore, we blocked both anti-H3 and anti-L1 IgGs simultaneously in serum from immunized mice. No reduction in VACV neutralizing antibody activity was observed (Fig. 9N).

**Anti-L1 IgG affinity purification.** The combined results of the L1 blockade experiments and L1 reverse immunoprecipitation depletion experiments (Fig. 9) suggested that even when anti-L1 antibodies are present in human vaccinees, the anti-L1 antibodies are not a major component of VACV neutralizing antibody activity. To resolve whether or not L1 is a neutralizing antibody target in smallpox-vaccinated humans, we developed an L1 affinity purification column strategy (Fig. 10) analogous to our earlier H3 work (Fig. 8). Plasma samples from two individual vaccinated donors (donors #3 and #4) were sequentially purified via the rL1 column (Fig. 10A to G and H to N, respectively). Flowthrough fractions from donor #3 plasma were 100% depleted of anti-L1 IgG (Fig. 10A to C), and the depletion was specific (Fig. 10B). The column was washed extensively to remove nonspecifically bound proteins (Fig. 10A to C), and then anti-L1 IgG bound to the column was eluted (Fig. 10A to C). The eluted protein was pure anti-L1 IgG, since no other specificities were detected by VACV microarray ELISA (Fig. 10C). Given that the levels of anti-L1 IgG in the plasma of vaccinee #3 were relatively low, limited amounts of anti-L1 IgG were recovered after affinity purification (4 ml of 0.35 µg/ml) (Fig. 10E). Nevertheless, purified human anti-L1 IgG exhibited clear VACV neutralization activity with good potency (Fig. 10D and F). Plasma from the same donor could be tested to determine what percentage of the VACV neutralization was contributed by anti-L1 antibodies. Neutralization assays were performed on L1-depleted (flowthrough) and undepleted (whole plasma) plasma samples. VACV neutralization by L1-depleted samples and that by undepleted samples were indistinguishable (Fig. 10G).

These experiments were repeated with a second donor (donor #4) (Fig. 10H to N), who had the strongest anti-L1 IgG response of any vaccinee in the cohort (Fig. 3). Anti-L1 IgG was efficiently isolated from plasma from this donor (Fig. 10H to J), and the anti-L1 IgG exhibited VACV neutralization specific activity (0.16 µg/ml) (Fig. 10M) similar to that of the first donor (0.27 µg/ml) (Fig. 10F). VACV neutralization assays were also performed on L1-depleted (flowthrough) and undepleted (whole plasma) plasma samples. VACV neutralization by L1-depleted plasma and that by undepleted plasma were indistinguishable (Fig. 10N). A direct comparison of the VACV neutralizing antibody titers of plasma from donors #3 and #4 before and after anti-L1 IgG depletion is shown in Fig. 10O. No difference was detectable.

## DISCUSSION

The smallpox vaccine is widely considered the gold standard of human vaccines, yet the key MV antibody targets in humans have remained unclear. Our approach to identifying the relevant neutralization target (or targets) was to determine the

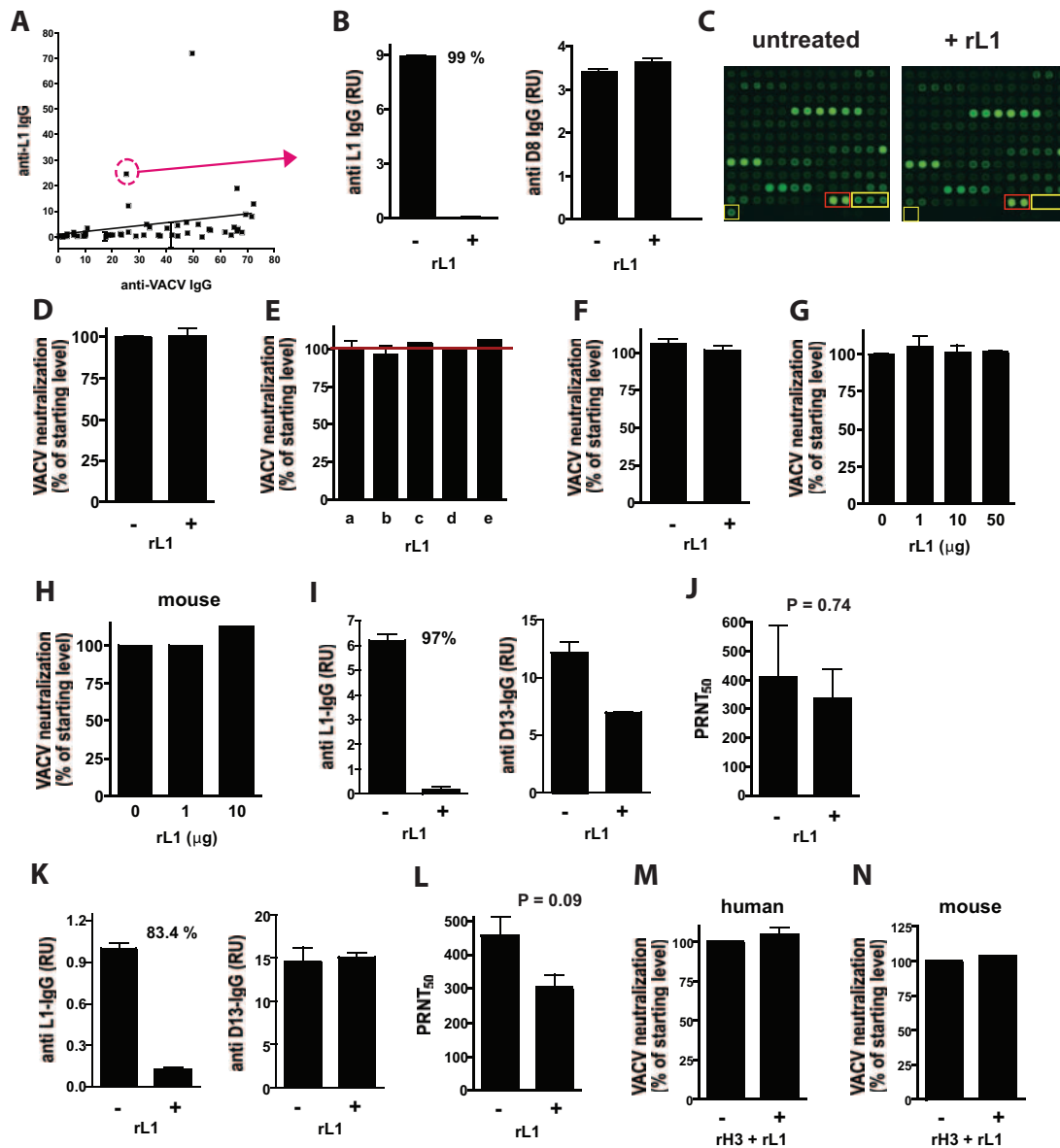


FIG. 9. Blocking or depleting human anti-L1 IgG. (A) Anti-L1 IgG graphed against total anti-VACV IgG, reproduced from Fig. 3B, with donor of interest circled. (B and C) Plasma blocked with the rL1 protein (10  $\mu$ g) and tested for efficiency and specificity of the blockade. (C) Raw microarray data. L1 protein spots (yellow box) and whole VACV lysate spots (red box) in untreated plasma (plasma alone) or blocked plasma (+ rL1). (D) VACV neutralizing antibody levels with or without rL1 blockade (percentage of starting level). Error bar indicates SEM for each condition. Data are representative of numerous experiments. (E) Percentage of VACV neutralizing antibody activity for each of five L1 seropositive human donors (a to e) measured in plasma samples after preblocking with the rL1 protein. The red line indicates 100% of starting VACV neutralization activity in untreated plasma from each individual vaccinee. (F) Data averaged for all six donors tested. (G) Quantitation of VACV neutralizing antibody activity in plasma samples from a smallpox vaccinee preblocked with different amounts of the rL1 protein (0, 1, 10, and 50  $\mu$ g). (H) VACV neutralizing antibody activity (percentage of initial level) in VACV-infected mice measured before (–) and after (+) anti-L1 blockade. (I) Depletion of anti-L1 IgG from the plasma of smallpox vaccinee-immunized humans by reverse immunoprecipitation (Ni-Sepharose plus His-rL1 plus plasma). (J) VACV neutralizing antibody titers ( $\text{PRNT}_{50}$ ) measured before (–) and after (+) anti-L1 antibody depletion. (K) Depletion of anti-L1 IgG from the plasma of a vaccinee by reverse immunoprecipitation, with the binding order changed (His-rL1 plus plasma plus Ni-Sepharose). (L) VACV neutralizing antibody titers ( $\text{PRNT}_{50}$ ) measured before (–) and after (+) anti-L1 antibody depletion in panel K. (M) Average VACV neutralizing antibody activity (percentage of initial level) for nine H3- and L1-seropositive human donors, measured in plasma samples before (–) and after (+) combined blocking with 10  $\mu$ g of the rH3 and rL1 proteins. (N) VACV neutralizing antibody activity (percentage of initial level) in VACV-infected mice measured before (–) and after (+) anti-H3 and -L1 combined blockade. Each data set is representative of multiple experiments. Error bars indicate SEM for each condition.

complete antigenic profile of the smallpox vaccine antibody responses of a cohort of 50 donors and then perform large-scale correlation analysis of titers of neutralizing antibody to each of the vaccinia virus MV surface proteins. The results

were encouraging. Anti-H3 IgG exhibited the best correlation with neutralizing antibody activity, which was consistent with the known ability of anti-H3 to neutralize VACV (13, 35) and protect mice from a lethal VACV infection (13). Furthermore,

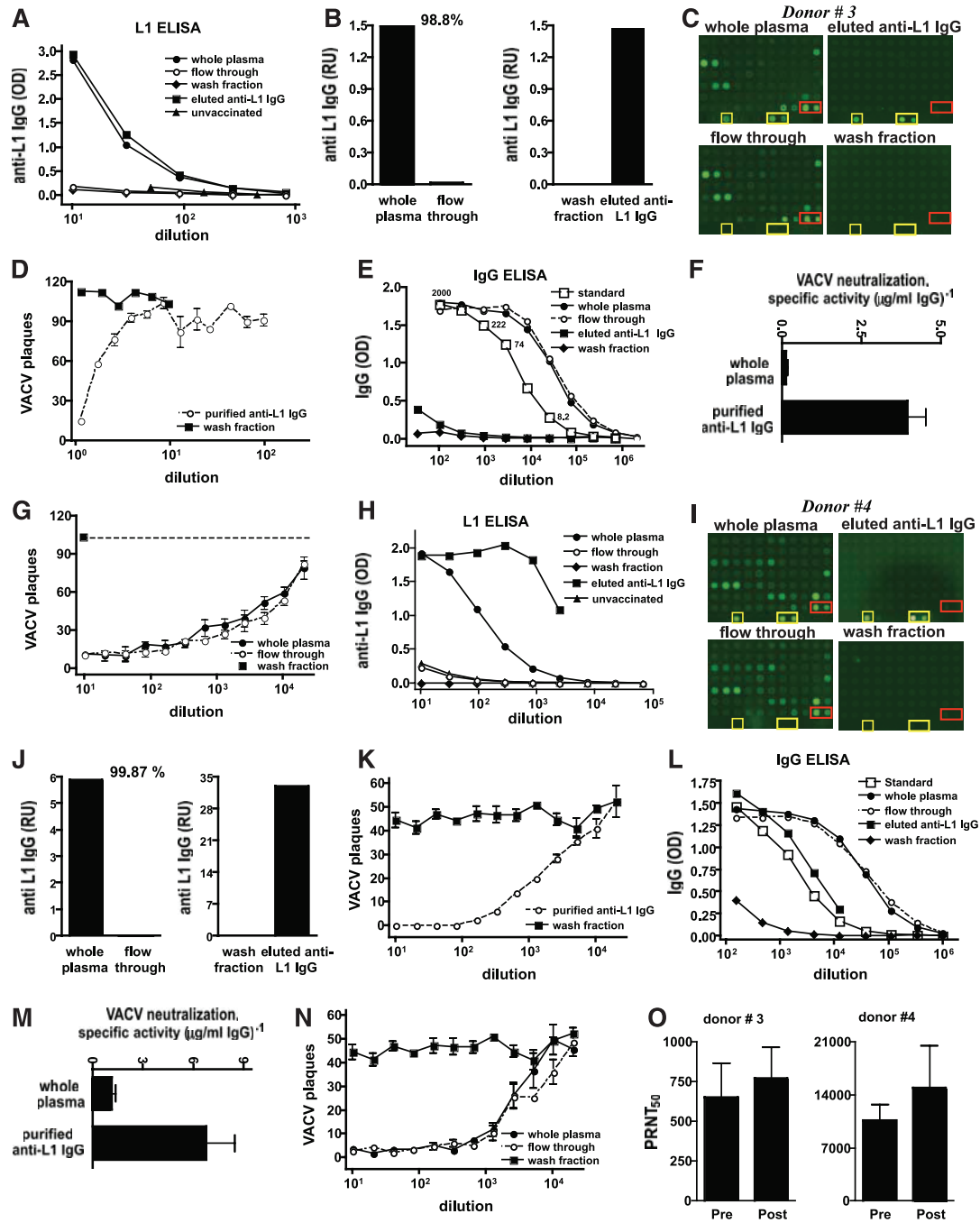


FIG. 10. Affinity purification of human anti-L1 antibodies from smallpox vaccinees. Antiviral antibodies from human smallpox vaccinee donor #3 (A to G) or donor #4 (H to O) were examined after depletion or purification of anti-L1 IgG using affinity column chromatography. Anti-L1 IgG in fractions was quantified by L1 ELISA (A) or protein microarray ELISA (B and C). (B) Specificity of the depletion and purification of anti-L1 IgG from the plasma sample was determined by VACV protein microarray ELISA. (C) Raw microarray data showing the L1 protein (yellow box) and positive control (total VACV lysate, red box). (D) VACV neutralizing antibody activity of purified human anti-L1 IgG. (E) Absolute concentrations of IgG in fractions were determined by IgG ELISA, using an IgG standard (open squares). (F) VACV neutralization-specific activity. (G) VACV neutralizing antibody activity of anti-L1-depleted plasma (“flow through”) or untreated plasma (“whole plasma”). (H to O) Anti-L1 purification and analysis from donor #4. (H) L1 ELISA. (I) Protein microarray data, as per panel C. (J) Anti-L1 IgG levels were quantified in whole plasma and flowthrough fractions (left) or in wash fractions versus eluted anti-L1 IgG (right). (K) VACV neutralizing antibody activity of purified human anti-L1 IgG. (L) Human IgG ELISA. (M) VACV neutralization-specific activity of purified anti-L1 IgG from donor #4. (N) VACV neutralizing antibody activity of anti-L1-depleted plasma (“flow through”) and untreated plasma (“whole plasma”) samples from donor #4. (O) VACV neutralizing antibody titers (PRNT<sub>50</sub>) determined for samples from human smallpox vaccinee donor #3 and donor #4 using whole plasma (precolumn [Pre]) or anti-L1-depleted plasma (postcolumn [Post]). All data are representative of multiple experiments. Error bars indicate SEM for each condition.

H3 was the immunodominant neutralizing antibody target as measured by seroprevalence in vaccinees (Table 1). We also detected positive correlations between virus neutralization activity and anti-D8, anti-A27, anti-A14, and anti-L1 IgG responses. These results were also logical, since D8, A27, and L1 are known neutralizing antibody targets in mice (29, 30, 51, 61). While anti-L1 IgG correlated with neutralizing antibody titers, the correlation was weak, which was in line with the observation that very few vaccinees make significant antibody responses to L1 (Table 1; Fig. 3). The correlations of anti-A14 and anti-D13 responses with neutralization activity were surprising and are worth detailed examination in the future.

While anti-H3 was the immunodominant response in terms of seroprevalence and correlation to neutralization activity, it was not determined whether H3 was the primary, or most important, neutralizing antibody target recognized. Anti-H3 IgG was prevalent, but was it singularly responsible for the majority of the VACV neutralization activity? The serological analysis suggested that anti-H3 IgG was not singularly responsible for virus neutralization in human vaccinee for several reasons: (i) anti-H3 IgG was detected in only ~75% of vaccinees (Table 1); (ii) while anti-H3 IgG was positively correlated with virus neutralization activity ( $P < 0.0001$ ), the accuracy of the anti-H3 IgG-PRNT<sub>50</sub> relationship was a moderate  $r^2$  value of 0.41 (Fig. 2); Putz and colleagues have also published data with an almost identical  $r^2$  value for their VACV Lister cohort (45) (see Table S1 in the supplemental material); (iii) neutralization activity correlations were detected for additional MV surface antigens (Fig. 2); and (iv) a large degree of heterogeneity was observed in the MV surface antigens recognized by different vaccinees (Fig. 3 to 5) (see Fig. S2 in the supplemental material), with L1 being the most extreme example, implying that there was no stereotypic smallpox vaccine neutralizing antibody response “signature.”

Direct examination of the functional relevance of anti-H3 and anti-L1 IgG in human responses to the smallpox vaccine produced surprising and complex results. Blocking of anti-H3 IgG with free rH3 or anti-L1 with rL1 resulted in no impact on the VACV neutralizing antibody activity (Fig. 6 and 9). Similar free protein H3 and L1 blockade results were recently reported by Putz and colleagues (45). We were skeptical of our results because of general concerns about the inclusion of large molar excesses of free viral receptor (H3 or L1) necessary in the experiments and the fact that the anti-H3 and anti-L1 antibodies were still physically present in the neutralization reactions. Therefore, we employed a series of three experimental approaches that physically removed the antibodies of interest (anti-H3 or anti-L1) from the plasma samples: (i) reverse immunoprecipitation; (ii) reverse immunoprecipitation with pre-complexed Ig and recombinant protein; and (iii) affinity column chromatography. In all cases, these experiments were done using multiple donors to eliminate the possibility that an unusual sample was being used. In all cases, complete removal of the anti-H3 or anti-L1 IgG was confirmed, and yet no significant reduction in VACV neutralizing antibody activity was measurable. These results perplexingly implied that the human anti-H3 and anti-L1 antibody responses were nonneutralizing in most vaccinees, which would be a surprising reversal from the published data on anti-H3 and anti-L1 neutralizing antibody responses in mice.

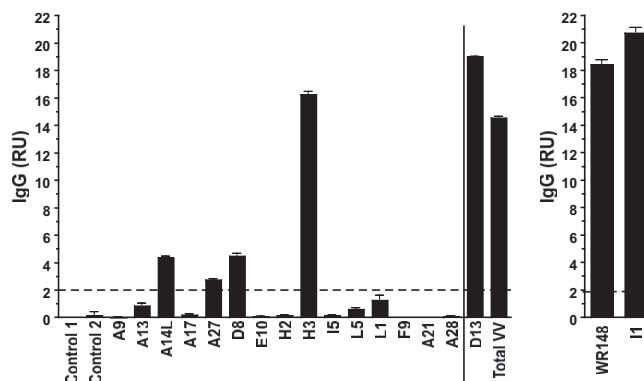


FIG. 11. VIG: IgG specific to known and potential VACV MV surface antigens. Two negative control antigens are shown (leftmost columns). VACV lysate was used as a positive control. Responses to VACV antigens WR148 and I1 are also shown. Dashed line, limit of detection. Error bars represent the full range of duplicate samples.

Given the surprising results, it was necessary to directly isolate human anti-H3 and anti-L1 IgG from individual vaccinees and determine whether the antibodies possessed significant VACV neutralization activity. To perform these experiments in a quantitative manner, affinity column chromatography was utilized. Anti-H3 IgG isolated from two different donors exhibited substantial VACV neutralization, with a PRNT<sub>50</sub>-specific activity of 0.5 to 0.625  $\mu\text{g/ml}$ . Comparable results were obtained from a parallel series of experiments examining affinity-purified human anti-L1 IgG (specific activities of 0.16 to 0.27  $\mu\text{g/ml}$ ). Nevertheless, depletion of the anti-H3 or anti-L1 IgG from these plasma samples did not significantly reduce the VACV neutralizing antibody activity. The data proves that there are at least three VACV MV neutralizing antibody targets recognized in vaccinated humans. While it is not proven, our data suggest that there are at least five neutralizing antibody targets recognized by some vaccinees: H3, L1, A27, D8, and A14 (Fig. 2 and 5) (see Fig. S2 in the supplemental material). Our serological analysis of VIG shows clear IgG responses to H3, A27, D8, D13, and A14, with low-level responses to L1, A13, and possibly L5 (Fig. 11). A report on affinity purification of anti-A27 IgG from VIG (made from pooled human plasma) was very recently published by Weiss and colleagues, and they reported that human anti-A27 IgG exhibits VACV neutralization activity (21), confirming our prediction (Fig. 2B). Importantly, they observed no reduction in VIG neutralizing antibody activity in anti-A27 IgG-depleted material (21). Putz and colleagues detected a modest reduction after blocking with the free A27, H3, or L1 protein (45); it is unclear whether the differences are due to the experimental techniques or to other considerations, such as the different vaccine strains used. As detailed in Fig. 6 to 10, we utilized multiple experimental techniques and samples from a variety of vaccinees and observed no reduction in neutralization activity after depletion of anti-H3 or -L1 antibodies. Altogether, our data indicate that immunodominance of smallpox vaccine surface antigens as measured by ELISA does not translate to immunodominance of surface antigens by neutralization activity.

**Dominant impact of redundant neutralizing antibody responses.** There are two possible interpretations of our overall

results. One, we did not identify the immunodominant neutralizing antibody target in vaccinated humans because the protein target was not represented in our study. While we have done our best to test the known MV surface molecules, there have been more recently identified MV surface proteins that we did not test (G9 and heavily disulfide-bonded A16 [42, 43]), as well as molecules recently reported to be exposed on the surface of MV (WR148 [8]), and we cannot exclude the possibility that one or more of the recombinant viral proteins we have used was imperfectly folded. A related hypothesis is that the major neutralizing antibody target is a multiprotein protein complex, with epitopes present only in the context of the complete complex. Therefore, it is formally possible that a new (or still uncharacterized) MV surface molecule is the primary neutralizing antibody target in vaccinated humans or that the response was hidden within the set of proteins we tested. We lean away from this option, because the evidence we have of extensive heterogeneity (both variability and diversity) of antibody targets observed (Fig. 3 to 5; Table 1) (see Fig. S2 in the supplemental material) supports a proposal where there is no singular primary antibody target in vaccinees. Between vaccinees we observed such extensive heterogeneity in the antibody responses to surface targets recognized that it seems unlikely that variability would not be observed for other putative MV targets that were not directly tested.

A second possible interpretation of these results is that there is a highly nonlinear, synergistic relationship between the neutralizing antibody concentration and virus neutralization activity, such that concentrations of antibodies below the limit of detection in this study were still physiologically relevant in neutralization. With this model in mind, we reexamine a donor such as the one represented in Fig. 5A, who has a strong anti-H3 response and a moderate anti-D8 response but no other detectable responses. Anti-H3 neutralizing antibodies were fully depleted from this donor (Fig. 6A, 7B, 7L, and 8K), yet no reduction in the neutralizing antibody titer was observed (Fig. 6D, 7G, and 8O). We now interpret this result to mean that the antibody responses to several of the MV surface proteins are functionally redundant and the anti-D8 response fully compensated for the loss of anti-H3 in conjunction with responses to additional specificities below our stringently defined limit of detection (perhaps H2, I5, L1, and A28; Fig. 5A). These varied specificities potentially synergize to make a highly redundant “safety net” of neutralizing antibody activity. This is a speculative model, but it is consistent with our available data. We can directly observe antibody responses to five or more potential neutralization targets in some vaccinees (e.g., see Fig. 4C). Furthermore, we have directly demonstrated that vaccinees can make neutralizing antibodies to H3 and L1 (proving that there are at least three VACV MV neutralizing antibody targets recognized in vaccinated humans), and A27 is now a known human neutralizing antibody target (21), and our correlation analysis further implicates additional neutralizing antibody targets, including D8, which is a known neutralizing antibody target in animal models.

In addition, we also showed that blocking anti-H3 or anti-L1 antibodies—or both—in serum from VACV-immunized mice did not significantly reduce the VACV neutralizing antibody activity of the serum samples (Fig. 7K, 9H, and 9N), indicating

that a highly redundant “safety net” of neutralizing antibody responses is also generated in the mouse model system.

Altogether, these data indicate that the smallpox vaccine succeeds in generating strong neutralizing antibody responses in vaccinees not by eliciting a stereotypic response to a single key antigen but instead by driving development of neutralizing antibody to multiple different viral proteins, resulting in a “safety net” of highly redundant neutralizing antibody responses, the specificities of which vary from individual to individual. We propose that this is a fundamental attribute of the smallpox vaccine, accounting for the ability of this vaccine to consistently elicit strong neutralizing antibody responses in very different human populations, by providing multiple neutralizing antibody targets. In the field of vaccinology, this can be interpreted as a cautionary tale about subunit vaccines. While they are appealing in their simplicity, subunit vaccines unfortunately may not reproduce the diversity of desirable targets which allow for a safety net of multiple specificities, since this appears to be a cornerstone attribute of the smallpox vaccine-elicited immune response.

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