

obtained from vaccines and compared with the specific vaccinia proteins recognized by each serum. Antibody recognition of the proteins C3L, I1L, and A33R correlated with the virus-neutralizing titers obtained from primary vaccinated individuals. Antibody binding to the putative DNA-binding virion core protein I1L exhibited the greatest correlation with virus-neutralizing titers, suggesting the importance of this newly detected antigen in directing protective immunity.

**[0264]** An essential subset of vaccinia proteins recognized by antibodies from vaccinated humans has been identified. The identification of these antigens was facilitated by the development of a vaccinia proteome microarray comprised of purified recombinant proteins that were produced by eukaryotic-cell expression. These proteins are important biomarkers of vaccinia immunity and potential targets for the development of new orthopoxvirus vaccines. The vaccinia proteins A27L, D13L, I1L, and H3L were recognized by antibodies from the majority of vaccinated subjects, while A33R, H5R, and C3L were bound by antibodies from over 25% of the vaccines. Antibody binding to the C3L, I1L, H5R, and D13L was exquisitely dependent on vaccination, as antibody binding to these antigens did not occur with sera from nonvaccinated individuals.

**[0265]** These results suggest that the primary antibody response to individual vaccinia proteins varies from individual to individual while the total number of proteins recognized by antibodies is only slightly altered by secondary vaccination. Proteins encoded by approximately 97 vaccinia ORFs were not included in the proteome microarray due to problems with protein expression. If it is assumed that these additional proteins have the same likelihood of antibody recognition as the proteins examined in the current microarray, then perhaps five more antigens may be included, resulting in a total of about 5% of the vaccinia proteome associated with antibody responses. The number of antibody-recognized proteins may increase if the untested proteins are inherently more antigenic. A comparison of all sera tested indicates that an array consisting of the vaccinia proteins A27L, D13L, I1L, H3L, A33R, H5R, C3L, and I3L may be sufficient for monitoring and evaluating antibody immunity to smallpox. All of the vaccinia proteins in this panel are represented by homologous or identical polypeptides present within the variola major and minor viral proteomes. In addition to the vaccinia-specific responses, antibodies that bound the arrayed proteins O2L and H7R were present in sera from several individuals, and this recognition pattern was independent of vaccination.

**[0266]** A recent report described a protein array that was used to measure antibody responses to vaccinia virus (Davies, et al., 2005(a), supra). The unsequenced gene clones from vaccinia were expressed in *E. coli* and used to create a microarray based on crude, unpurified, recombinant proteins. Several vaccinia proteins were specifically recognized by serum antibodies in this previous study, some confirmed by our analysis, though considerable background binding of antibodies was noted due to the preponderance of contaminating *E. coli* proteins. However, additional proteins reported here and elsewhere (Galmiche, et al., *Virology* 254:71-80, 1999) were not detected by immune sera in the recent report in part because the bacterial expression system used for the preparation of the microarray elements resulted in incomplete post-translational modifications of the vaccinia products. Although it is difficult to assess correct folding of microar-

rayed proteins, catalytic function was retained by several of the enzymatic vaccinia proteins on the arrays used in this study.

**[0267]** The antibody-binding proteins detected by microarray are significant biomarkers for measuring antibody responses to vaccinia, yet not all may be essential for immunity. For example, antibodies against A33R do not neutralize infection by EEV. However, immunization with A33R, a protein required for the formation of actin-containing microvilli and efficient cell-to-cell spread of vaccinia virus, protected mice against a lethal virus challenge, suggesting that this protein may be more important for CTL responses. It has been reported that antibody responses remain stable for up to 75 years after vaccination, whereas T-cell immunity slowly declines, with a half-life of 8-15 years. A comparison of vaccinia protein recognition with previously published data for T-cell recognition indicates that I1L, H3L, and A27L stimulate T-cell immunity among individuals expressing the high-frequency MHC class I allele HLA-A\*0201, while C3L and I3L are also reported to be T-cell antigens. It may be possible to routinely evaluate biomarkers for both cellular and antibody-mediated immunity as high-throughput methods for evaluating T-cell responses become available. Further complexity in antibody-response profiles is influenced by expression-phase variation in viral antigens presented during the infective cycle. Antibody depletion experiments previously demonstrated that the EEV surface protein B5 contributes to EEV neutralization in vaccinated humans, whereas A27L and H3L are targets for IMV-neutralizing antibodies.

**[0268]** The present vaccinia proteome microarray will be useful for evaluating immunity to new vaccines. The highly attenuated vaccinia virus strain, NYVAC (vP866), was derived from a plaque-cloned isolate of the Copenhagen vaccine strain by the deletion of 18 ORFs, including the complement 4b binding protein C3L. These results indicate that C3L is an antigen recognized by a significant number of individuals receiving the DRYVAX® vaccine, suggesting the contribution of this protein to protective immunity against smallpox. In addition, antigenic variations between proteins produced by smallpox virus and attenuated vaccines have not been sufficiently addressed. For example, the vaccinia virus complement control protein is nearly 100-fold less potent than the homologous smallpox inhibitor of complement enzymes at inactivating human C3b, contributing to the lower virulence of vaccinia compared to variola virus. Antibody recognition of complement control protein and other virulence factors may also differ between pathogen and vaccine.

**[0269]** The vaccinia proteome microarray described herein represents an important advancement over previously reported arrays in that the identity of each clone was confirmed by sequencing, the majority of all predicted proteins encoded within the viral genome were purified and arrayed, and eukaryotic cell expression increased the likelihood of natively folded proteins. Though antibody binding may not require native folding for many of the vaccinia proteins, high-content arrays of functional proteins provide a high-throughput tool for evaluating protein-protein interactions and biological activities of all elements contained within the viral proteome. Thus, a full inventory of vaccinia proteins required for optimal protection against smallpox will speed the development of safer, better-defined vaccines and will contribute substantially to devising new strategies for therapy.

#### Example 7

##### Microarray-Based Anthrax Model

**[0270]** This example describes the principles for designing an in vivo rabbit model for anthrax vaccine, antimicrobial and