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Short communication

Profiling of the antibody response to attenuated LC16m8 smallpox vaccine using protein array analysis

Akiko Eto [a], Masanori Fujita [b], Yasumasa Nishiyama [c,1], Tomoya Saito [a], Douglas M. Molina [d], Shigeru Morikawa [e,2], Masayuki Saijo [f], Yasuhiko Shinmura [g], Yasuhiro Kanatani [a,*]

[a] Department of Health Crisis Management, National Institute of Public Health, 2-3-6 Minami, Wako-shi, Saitama 351-0197, Japan
[b] Division of Environmental Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa-shi, Saitama 359-8513, Japan
[c] Health Care Center, Japan Self-Defense Forces Central Hospital, 1-2-24 Ikeziri, Setagaya-ku, Tokyo 154-8532, Japan
[d] Antigen Discovery, Inc., Irvine, CA, United States
[e] Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
[f] Department of Virology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
[g] KM Biologics Co., Ltd, Kyokushi, Kikuchi, Kumamoto 860-8568, Japan

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ABSTRACT

Concerns about bioterrorism and outbreaks of zoonotic orthopoxvirus require safe and efficacious smallpox vaccines. We previously reported the clinical efficacy and safety profiles of LC16m8, a live, attenuated, cell culture-derived, smallpox vaccine, examined in over 3000 healthy Japanese adults with various vaccination histories. In this study, serum of approximately 200 subjects pre and post LC16m8 vaccination were subjected to a vaccinia virus-specific protein array to evaluate the proteome-wide immunogenicity. The relationships between antigen-specific antibodies and plaque reduction neutralization titers were analyzed. LC16m8 induced antibodies to multiple vaccinia antigens in primary-vaccinated individuals and yielded effective booster responses in previously vaccinated individuals, demonstrating similar antibody profiles to those reported for other vaccinia virus strains. Several immunodominant antigens were indicated to be important for neutralization of the intracellular mature virion. The similarity of antibody profiles between LC16m8 and other smallpox vaccine strains supports the immunogenicity and protective efficacy of LC16m8.

1. Introduction

LC16m8, a live, attenuated, cell culture-derived smallpox vaccine, was developed and licensed in Japan in the 1970s, but was not used for the routine smallpox vaccination at the time [1,2]. The World Health Organization (WHO) declared eradication of smallpox in 1980 [3,4], and routine smallpox vaccination was halted. However, the need for smallpox vaccination was reconsidered in the early 2000s for possible bioterrorism [1–4]. Moreover, outbreaks of zoonotic orthopoxvirus infections raised concerns [3,4]. Accordingly, LC16m8 production was restarted and the vaccine has been stockpiled by the Japanese government [1]. In 2013, the Strategic Advisory Group of Experts on Immunization recommended LC16m8 as a WHO stockpile of smallpox vaccines [5]. Because first-generation smallpox vaccines may cause rare but severe adverse events, safer vaccines have been sought in the absence of endemic outbreaks [3,4].

We have previously reported the clinical and immunological responses to LC16m8 vaccination in over 3000 healthy Japanese adults [6]. No severe adverse events and high levels of vaccine take and seroconversion were observed, confirming the safety and efficacy of the vaccine [6,7]. A comparative clinical phase I/II study conducted in the United States with the Dryvax smallpox vaccine, and animal model studies have also demonstrated the safety and efficacy of LC16m8 [8–10].

Abbreviations: WHO, World Health Organization; EEV, extracellular enveloped virion; IMV, intracellular mature virion; WR, Western Reserve strain; PRN, plaque-reduction neutralizing.

* Corresponding author at: Department of Clinical Pharmacology, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan.
E-mail address: kanatani.yasuhiro.f@tokai.ac.jp (Y. Kanatani).
[1] Present address: Health and Medical Division, Bureau of Personnel and Education, Ministry of Defense, 5-1 Ichigaya-honmura-cho, Shinjuku, Tokyo 162-8860, Japan.
[2] Present address: Faculty of Veterinary Medicine, Okayama University of Science, 1-3 Ikoinooka, Imabari City, Ehime 794-8555, Japan.

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However, humoral immunity plays a pivotal role in defense against smallpox virus [3,4], and use of protein microarrays enables elucidation of the overall antibody production profile [11]. Therefore, in the present study, we assessed the antigenicity of LC16m8-vaccinated serum in primary and previously vaccinated individuals, using a vaccinia virus-specific antigen-loaded protein array [11].

2. Methods

2.1. Sera

Sera from humans vaccinated with LC16m8 were obtained in our previous study [6]. The protocol was approved by the institutional review board of the Self-Defense Forces Central Hospital, Tokyo (No. 16-004; August 20, 2004). Serum samples were collected before (day 0) and 30 days after vaccination and stored at −80 °C until further use.

2.2. Plaque reduction neutralization (PRN) test

The PRN titer data for sera against the Dryvax smallpox vaccine, obtained in the previous study [6], were used in the analysis performed in the study described here.

2.3. Protein array analysis

Serum antibody profiles of subjects from the second round of the vaccination program were examined using a protein array (Antigen Discovery Inc., Irvine, CA, USA), as previously described [11–16]. Briefly, Western Reserve (WR) antigens were expressed in an *in vitro* transcription/translation system (RTS100 *E. coli* HY kits, 5 Prime GmbH, Germany) and used without purification [11–16]. The presence of the expressed proteins was confirmed with polyhistidine and hemagglutinin tags at the N- and C-termini of the proteins, respectively [11]. Herein, antigens are described using the Copenhagen strain nomenclature. Some spots containing no virus-coding proteins (no insert or no vector) were used as negative controls ("No DNA control"). Slides were blocked in protein array blocking buffer (Maine Manufacturing, Sanford, ME, USA) for 30 min prior to serum probing.

Serological hybridization experiments were performed in triplicate. Sera were diluted 1:100 in blocking buffer containing 10% *E. coli* lysate. Bound antibodies in sera were visualized with biotinylated goat anti-human IgG Fcγ fragment-specific secondary antibody or biotinylated goat anti-human IgA + IgG + IgM (H + L) secondary antibody (both from Jackson ImmunoResearch Labs, West Grove, PA, USA) to detect IgG, followed by hybridization with streptavidin-PBXL3 (Columbia Biosciences, Frederick, MD, USA), and scanned using an autoloader-equipped GenePix 4300 instrument (Molecular Devices, Sunnyvale, CA, USA). As an anti-vaccinia positive control, vaccinia immunoglobulin (VIG) was also probed onto the array. The assays were performed at Antigen Discovery Inc. (Irvine CA. USA).

2.4. Data processing and normalization

Protein array construction differed slightly in each experiment in that the number of expressed antigens and controls varied. A total of 195 antigens were commonly included in all experiments and data for these antigens were used for analysis. Data for L1 and A17 were excluded because of insufficient measurements. To stabilize the variance of the data and to minimize array-to-array and experiment-to-experiment variation, the data set was normalized via vsn normalization on R platform (2.14.0) [15]. Data without a positive control signal were excluded before normalization. After normalization, the median value of the triplicate measurements was defined as the representative value for each sample. The resultant value was retransformed to the measured scale as described previously [15].

2.5. Study design and statistical analysis

Subjects were stratified into four groups based on age and vaccination history [6], as summarized in Table 1. Individuals with no clinical take in group A were not included in the analysis [6].

To assess the immune response to LC16m8, the mean signal intensity for each antigen was compared in pre- and post-vaccination sera in each group by paired *t*-tests (one-sided). The correlations between PRN titer against Dryvax smallpox vaccine [6] and signal intensities against anti- intracellular mature virion (IMV) membrane protein were examined. The level of antibodies based on the PRN titer were compared between high PRN titer ($\geq$32) (N = 38 for naive group and N = 222 for revaccinees) and low PRN titer (<32) groups (N = 44 for naive group and N = 84 for revaccinees) [4], by *t*-test. To generate heat maps, Multi Experiment View (Mev 4.8.0) Software (The Institute for Genomic Research [TIGR]) was used, as previously described [12]. The results for 10 subjects are shown for each group, placed in ascending order of PRN titer from left to right. The top 30 reactive antigens in group A are presented as indicated on the right side of the panel. The background measurement for each sample (i.e., no DNA control) was subtracted from the signal intensity for each antigen. Statistical analysis was performed using R (2.14.0) or JMP (version 9.0, SAS Japan).

3. Results

Fig. 1 shows pre- and post-vaccination signal intensities of the top 20 increased antibodies for groups A-D. Protein array data for the pre-vaccination sera of groups B-D represent the profiles of the pre-existing antibodies delivered by routine vaccination until the 1970s. The dominant residual antibodies included WR148, A4, D13, A10, and A56 for group B; WR148, D13, I1, A4, and H3 for group C; and I1, WR148, D13, H3, and A4 for group D. WR148 is a homolog of A-type inclusion protein, A4 is a membrane-associated core protein, D13 is a virion coat protein, A10 and I1 are core proteins, A56 is an extracellular enveloped virion (EEV) membrane protein, and H3 is an IMV membrane protein [17]. Fewer EEV membrane protein-targeting antibodies were detected. The antibody profiles did not differ markedly among the groups, though the magnitude of signal intensities was higher in groups C and D than in group B.

Upon LC16m8 vaccination, antigen-specific antibodies were induced in all groups. This increase was much higher in the re-vaccinated individuals, particularly in groups C and D, than in the naive group. In the naive population, the differentially recognized antigens among the pre- and post- vaccination included D13, WR148, D8, A13, A27, A10, I1, H3, A11, and A33. D8, A13, and A27 are antigens of the IMV membrane, and A33 is an antigenic component of the EEV membrane. The EEV/CEV antigens, A33, A56, A36, F13, and A34, were among the 15 most reactive antigens in this cohort. In revaccinees, the most reactive antigens included D13, D8, I1, A27, and WR148 for group B, and D13, WR148, I1, D8, and H3 for groups C and D. These profiles were similar among these groups and with those of the naive group. The results revealed that multiple antibodies were induced at various expression levels with different immunogenic specificities for each subject as shown in heatmap image (Fig. 2). The high and variable baseline of pre-vaccination serum in naive individuals (group A)

Table 1
Age group.

Group	Birth year	Age range at LC16m8 vaccination	Previous vaccination	
			Dose	Vaccine strain
A	1976–1984	20–27	0	–
B	1970–1975	28–33	1	Lister
C	1964–1969	34–39	2	Ikeda, Lister
D	1953–1963	40–51	3	Dairen, Ikeda, Lister

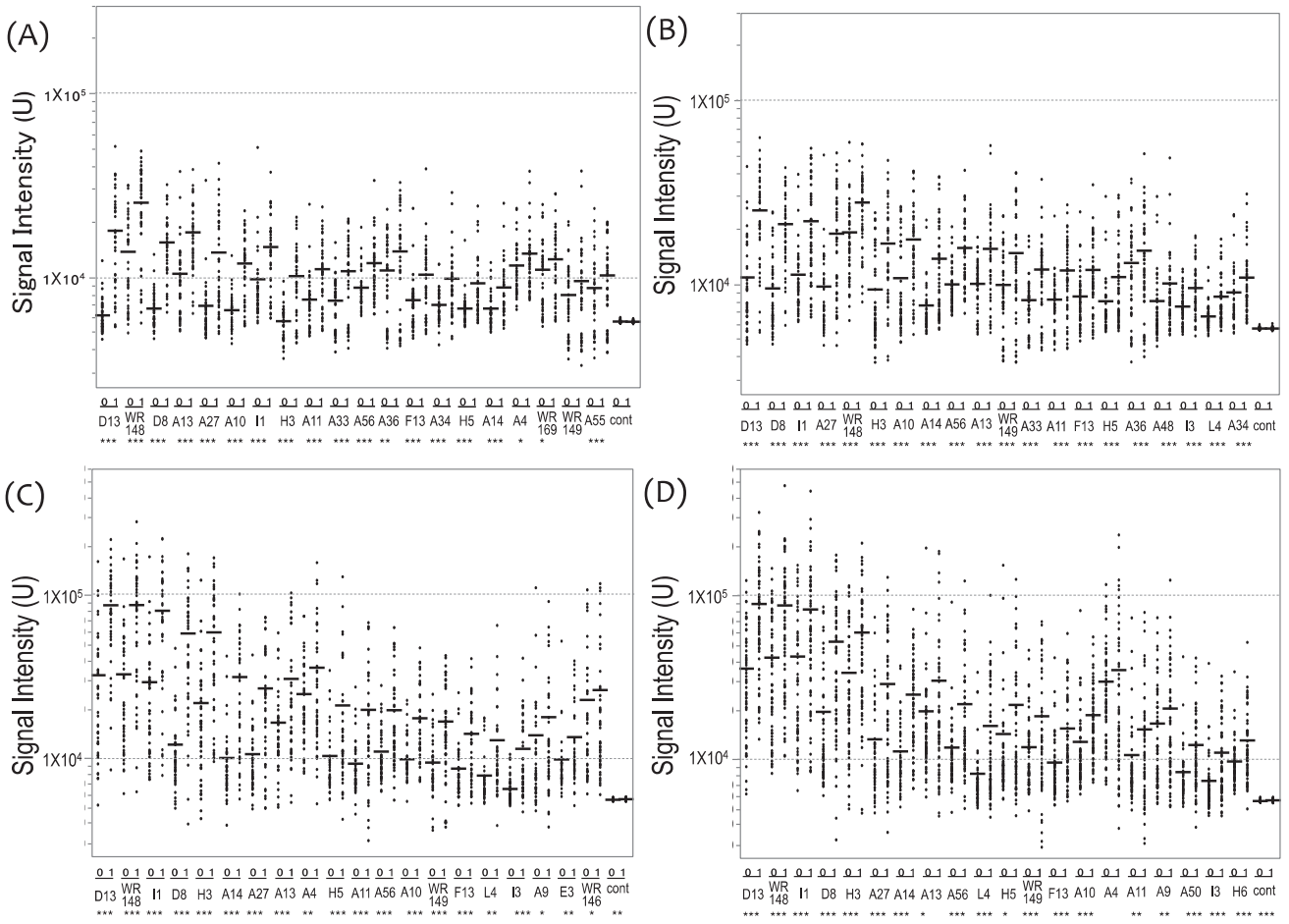

Fig. 1. Comparisons of average signal intensities pre- and post- vaccination against each antigen in groups A, B, C, and D. (A) Group A, (B) Group B, (C) Group C, and (D) Group D. Individual data points for pre-vaccination ("0") and post-vaccination sera ("1") were plotted. The bar indicates the average value. * indicates differentially expressed antibodies (*p < 0.05, **p < 0.005, ***p < 0.0005). The top 20 induced antigens are presented in each group.

could be due to non-specific binding or background noise, as previously reported [11,12].

In anti-vaccinia immunity, multiple antibodies are thought to be involved in neutralization [14,18]. To investigate such antigens, the correlation between the signal intensities for IMV antigens [17] and PRN titer were analyzed. In pre-vaccination sera, a modest or weak correlation was shown for anti-D8, -H3 -WR148, -A14, and -A27 antibodies (r = 0.4889, 0.4387, 0.3249, 0.2208, and 0.2167, $P < 0.05$, respectively). Upon vaccination with LC16m8, a weak correlation was observed for anti-D8 antibodies in the naive group (r = 0.3779, $P < 0.05$). In revaccinees, anti-WR149, -H3, -A27, -A14, and -D8 antibodies displayed weak correlations with PRN titer (r = 0.3426, 0.2891, 0.2776, 0.267, and 0.2601, respectively, $P < 0.05$).

A comparison of the mean signal intensities between the high (≥32) and low (<32) PRN titer groups showed that, among the IMV antigens, antibodies against H3, D8, A13, A14, WR148, and A27 were significantly higher in the high PRN titer group than in low PRN titer group both in the naive ($P = 0.0022$ for A14, < 0.0001 for the other 5 antigens) and revaccinated individuals ($P = 0.0155$ for A13, <0.0001 for other 5 antigens) (Fig. 3), suggesting the importance of these antibodies in the human immune response upon vaccination.

4. Discussion

LC16m8 induced a broad spectrum of antibodies in primary-vaccinated individuals and yielded effective booster responses in previously vaccinated individuals, with a profile similar to that of the naive cohort. The profile and manner of production of the antibodies were broadly similar to those identified for other smallpox vaccine strains and convalescent smallpox sera [11–16].

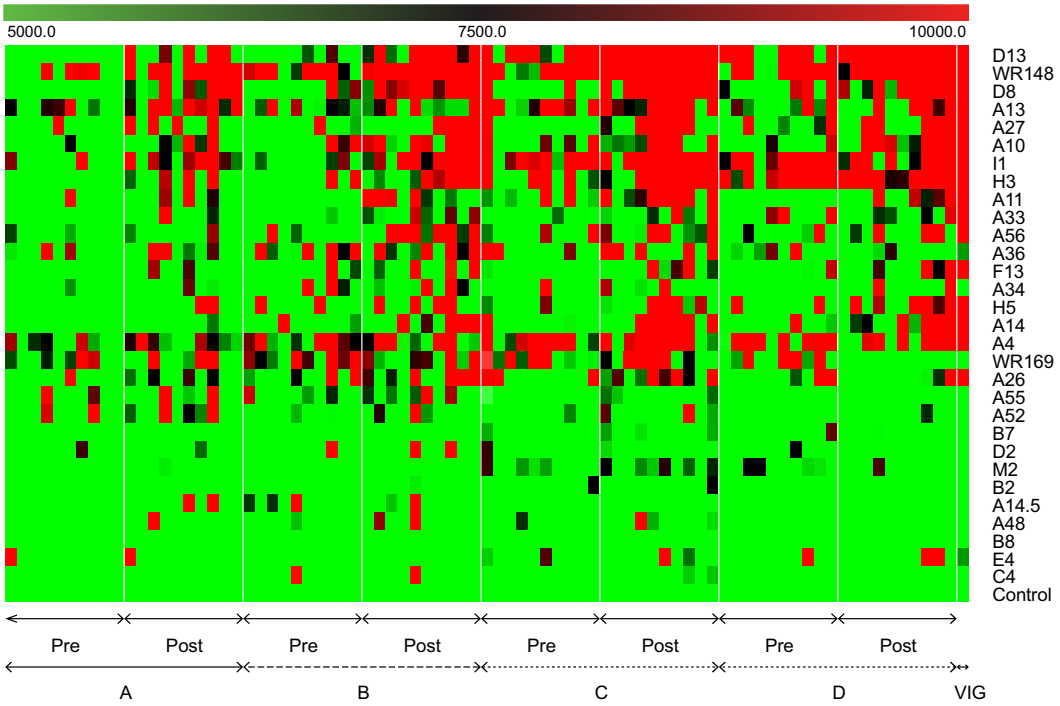

Fig. 2. Heat map image of subjects in each group pre and post LC16m8 vaccination. The subjects were selected systematically with equal interval rank according to the PRN titer, and there were 10 samples in each group. The results for the top 30 reactive antigens in group A are shown, as indicated on the right side of the panel. The rightmost column shows the result for VIG.

Antibodies yielded by the first-generation smallpox vaccines and maintained for decades were largely similar to those detected at 30 days post-vaccination for naive group, including neutralizing antibodies. However, the profile of pre-existing antibodies has slightly different trends compared to the profile at 30 days post-vaccination: non-membrane protein antibodies are more dominant (i.e., A4, D13, A10) and EEV-targeted antibodies are less prominent. These results are consistent with a previous study showing that anti-EEV neutralizing activity waned more rapidly than anti-IMV neutralizing activity over periods encompassing decades [3]. Our results showed the profile of long-term antibodies in a relatively large cohort with a clear vaccination history [6], without re-exposure to antigens.

The immunogenic similarity of LC16m8 and other smallpox vaccine strains is consistent with their highly homologous homologous sequences. Consequently, the antigens that were functionally important antigens for protection overlapped: our data indicated the importance of H3, D8, A13, A14, WR148, and A27, similar to that shown by previous results for the Dryvax smallpox vaccine [14].

The levels of antibodies against the EEV antigens were elevated in primary vaccinated individuals upon LC16m8 inoculation, although the magnitude of the increase was not striking (Fig. 1). These results reveal that LC16m8 EEV proteins are targeted as the major antigens by the human immune system. These attributes may contribute to the protective efficacy of LC16m8 [2,9,10], since the presence of neutralizing antibodies against the IMV and EEV proteins is important for establishment of immunity [3,4,18]. The frequency of responders to B5 was relatively low, as previously reported [19], probably due to a mutation in the protein. Although anti-B5 antibody production has been discussed in terms of the relevance of LC16m8 efficacy, several studies have shown that a LC16m8 or B5-deleted mutant virus confers protection in animal models [2,9,10,20] and that single antigens are not solely responsible for the protective immunity conferred by smallpox vaccines [3,4,14,18].

There were some limitations in the present study. First, the high and variable baseline values of antibodies were observed in non-immune serum, which was likely due to non-specific binding or background noise, as previously reported [11,12]. Second, we did not include vaccinia virus-irrelevant proteins as negative controls in the he protein array measurement, which would be preferable [16]. Third, data from several experiments were merged and normalized together, and we thus could not exclude a possibility that this procedure would affect the results.

We assume that the antibody profile induced by LC16m8 is consistent with its protective efficacy, with the reservation that protection was confirmed only in animal models. Together with studies indicating the safety and efficacy of LC16m8 [6–10,19], these findings have implications for vaccine development and vaccination strategies in the future.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Y.S. has been employed by the Chemo-Sero Therapeutic Research Institute and is employed by its succeeding company, KM Biologics Co., Ltd. All other authors report no potential conflicts of interest.].

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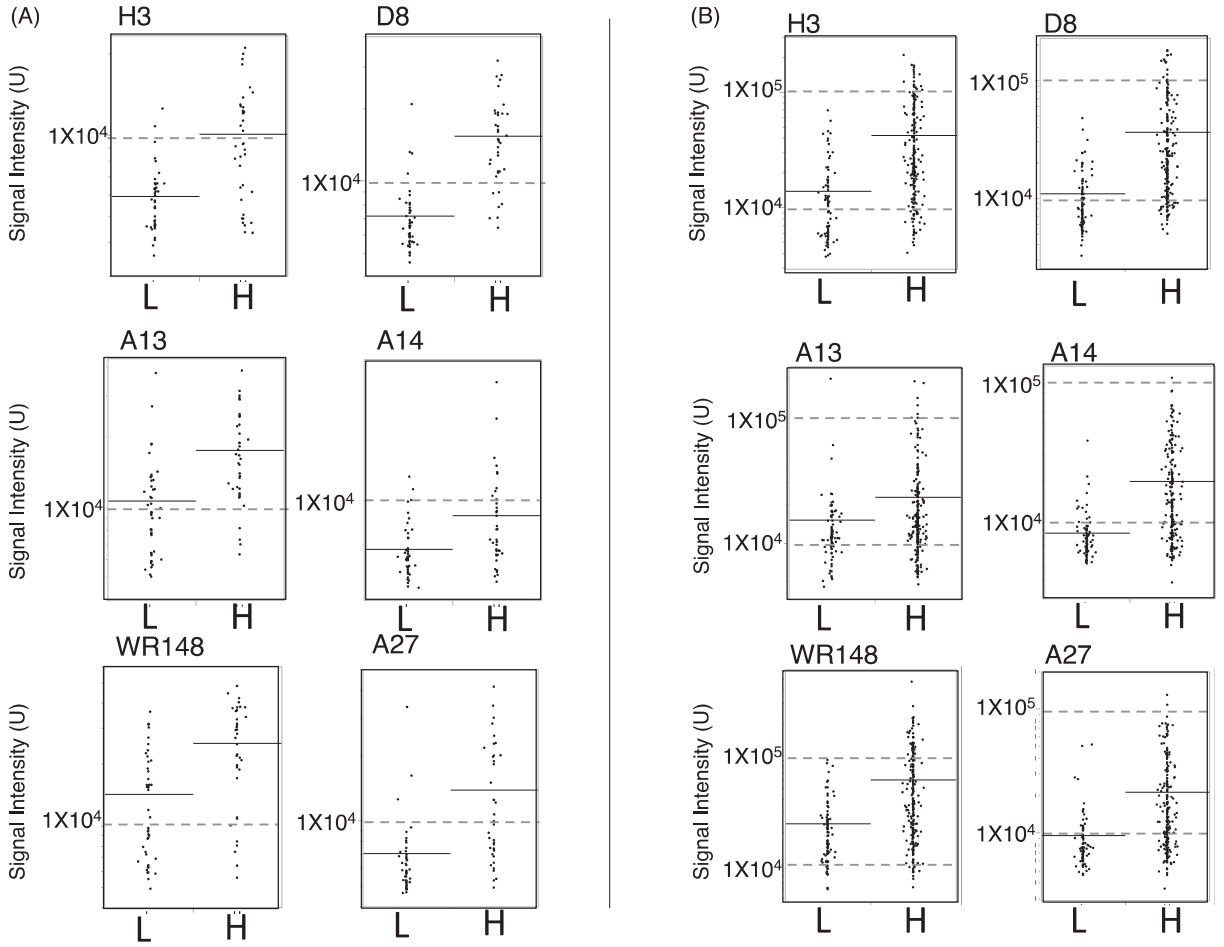

Fig. 3. Signal intensities in the PRN titer high (H) and low (L) groups. (A) Primary vaccinated group (group A) and (B) Previously vaccinated groups (groups B, C, and D). The bar indicates the average value. Six antigens of IMV membrane proteins are shown.

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Role of funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the paper for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Contributors' Statement

A.E. carried out data analysis, interpretation of the results, and drafted the initial manuscript; M.F. contributed to interpretation of the results; Y.N contributed to acquisition of data; T.S., S.M., and M.S. contributed to interpretation of the results; D.M carried out the proteomic measurements; Y.S. contributed to data acquisition and interpretation of the results, Y.K. acquired the funding and supervised this study. All authors reviewed and revised the manuscript, and approved the final manuscript as submitted.

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