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Improvement of Antibody Responses by HIV Envelope DNA and Protein Co-Immunization

Franco Pissani^{1,2,3,#,¶}, Delphine C. Malherbe^{3,#}, Jason T. Schuman⁴, Harlan Robins⁵,
Byung S. Park^{3,6}, Shelly J. Krebs^{2,3,¶}, Susan W. Barnett⁷, and Nancy L. Haigwood^{1,2,3,*}

¹Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland OR 97217

²The Vaccine and Gene Therapy Institute, Beaverton OR 97006

³Oregon National Primate Research Center, Beaverton OR 97006

⁴GE Healthcare, Life Sciences, Piscataway NJ 08854

⁵Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle WA 98109

⁶Department of Public Health and Preventive Medicine, Oregon Health & Science University, Portland OR 97239

⁷Novartis Institutes for Biomedical Research, Cambridge, MA 02139

Abstract

Background—Developing HIV Envelope (Env) vaccine components that elicit durable and protective antibody responses is an urgent priority, given the results from the RV144 trial. Optimization of both the immunogens and vaccination strategies will be needed to generate potent, durable antibodies. Due to the diversity of HIV, an effective Env-based vaccine will most likely require an extensive coverage of antigenic variants. A vaccine co-delivering Env immunogens as DNA and protein components could provide such coverage. Here, we examine a DNA and protein co-immunization strategy by characterizing the antibody responses and evaluating the relative contribution of each vaccine component.

Method—We co-immunized rabbits with representative subtype A or B HIV gp160 plasmid DNA plus Env gp140 trimeric glycoprotein and compared the responses to those obtained with either glycoprotein alone or glycoprotein in combination with empty vector.

Results—DNA and glycoprotein co-immunization was superior to immunization with glycoprotein alone by enhancing antibody kinetics, magnitude, avidity, and neutralizing potency. Importantly, the empty DNA vector did not contribute to these responses. Humoral responses elicited by mismatched DNA and protein components were comparable or higher than the responses produced by the matched vaccines.

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*Corresponding author: Oregon National Primate Research Center, Oregon Health and Science University, 505 NW 185th Avenue, Beaverton, OR 97006, Phone: (503) 690-5500, Fax: (503) 690-5569, haigwood@ohsu.edu.

#These authors contributed equally to this work.

¶Current address: U.S. Military HIV Research Program, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910

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Conclusion—Our data show that co-delivering DNA and protein can augment antibodies to Env. The rate and magnitude of immune responses suggest that this approach has the potential to streamline vaccine regimens by inducing higher antibody responses using fewer vaccinations, an advantage for a successful HIV vaccine design.

Keywords

HIV; Envelope-based vaccine; DNA+protein co-immunization; neutralizing antibodies

INTRODUCTION

The recent report of partial efficacy in the phase III RV144 trial underscores the challenge of designing HIV vaccines that can protect from infection. Effective vaccines may require complex regimens to elicit adaptive responses to multiple antigens. In RV144, prime-boost immunizations with recombinant ALVAC and gp120 proteins, including co-administration of these components for the last two immunizations, resulted in reduction of viral acquisition that was associated with antibodies directed to the HIV Envelope protein (Env) [1, 2]. Neutralizing antibodies (NAbs) can block SIV or SHIV infection in macaques [3–6] and appear to contribute to the control of post-infection viremia in HIV infected humans [7]. The strength of interactions occurring between polyclonal antibodies and antigen, termed antibody avidity, has recently emerged as a central feature of antibody-based vaccines [8, 9]. In addition, nonhuman primate (NHP) SIV challenge models have provided additional evidence that T cell-based vaccines can offer substantial viral control [10] but cannot prevent infection, in contrast to vaccines that include Env components [11, 12].

The vast variability and plasticity of Env are major obstacles to HIV vaccine design, and vaccines designed to elicit NAbs have resulted in antibodies with relatively narrow breadth and potency [13–18]. Prime-boost immunizations can increase the conformation dependence of antibodies [17] with the caveat of prolonged immunization schedules. These results emphasize the need for vaccines that rapidly elicit potent Env-specific antibodies that provide better coverage of antigenic variants. There is mounting evidence that indicates combining Env DNA and protein vaccine components may address this need. Indeed, we recently demonstrated that co-immunization with HIV-1 envelope DNA and trimeric protein accelerates the NAb response [19] and elicits T cell responses [20]. These findings have been extended by other groups who have found similar results of increased humoral responses in mice and macaques [12] as well as increased NAb breadth [21], but the contribution of each component has not been addressed yet. Here, in order to further characterize the *env* encoded-DNA plus gp140 protein co-immunization strategy, we used model Env immunogens from two different clades and parsed the contribution of the individual DNA and protein components by co-immunizing rabbits with either matched or mismatched subtype A and B immunogens. Our findings demonstrate that regardless of whether the immunogens were matched or mismatched, co-immunizations with DNA and protein enhanced the overall antibody response compared to immunizations with protein alone or empty vector plus protein. Importantly, our results further suggest that combining Envs derived from different sources may, in some cases, enhance antibody binding, avidity, and neutralization potency.

MATERIALS AND METHODS

Animals

Female New Zealand White rabbits (Western Oregon Rabbit Company) were housed at ONPRC; procedures were approved by the OHSU IACUC.

HIV-1 Env Immunogens and Rabbit Immunizations

Codon-optimized SF162 (subtype B) and motif-optimized [22]Q461e2TAIV (subtype A) gp160 DNA were cloned into pEMC*, and precipitated onto gold bullets to immunize rabbits intradermally by Gene Gun (Bio-Rad) [19, 23]. Recombinant trimeric gp140 proteins (50 µg; fully characterized in [13, 24]) mixed with an equal volume of polyethylenimine adjuvant (PEI, branched; Sigma-Aldrich), were concurrently delivered intramuscularly. Blood was collected every two weeks and sera were heat-inactivated.

Antibody Assays

Longitudinal binding antibody responses to SF162 and Q461e2TAIV trimeric gp140 were measured by kinetic ELISA [19] with chimpanzee IgG as standard. The avidity index to both antigens was determined as described [8] by endpoint ELISA with minor modifications. Avidity of sera was determined by calculating the midpoint antibody titer after treatment with 8M Urea compared to PBS for each antigen.

Surface Plasmon Resonance Assays

Antibody concentrations were determined on a Biacore T200 (GE Healthcare) at 25°C. SF162 and Q461e2TAIV trimers were immobilized at 20µg/mL in 10mM acetate buffer (pH=5.0) to flow cells 2 and 3 on a CM5 chip by amine coupling (8,860RU for SF162 and 10,930RU for Q461e2TAIV). 50µg/mL Protein A (Pierce) in 10mM acetate buffer (pH=4.5) was immobilized on flow cell 4 (2,330RU). The reference flow cell was activated and blocked with ethanolamine. Samples were diluted into HBS-EP+ buffer with 0.2mg/mL BSA. An antibody standard containing polyclonal antibodies to both Q461e2TAIV and SF162 was generated by determining the concentration of a high titer sample (injected at 5 and 100µL/min for 36s) using calibration-free concentration analysis (CFCA). The data were fit using $8.526 \times 10^{-11} \text{ m}^2/\text{s}$ as a translational diffusion coefficient for IgGs at 25°C. Experiments were performed at dilutions 1:100 and 1:1600 to determine Env-specific and total antibody concentrations respectively. This standardized sample was then used to create a calibration curve to determine the concentration for all other samples, which were tested at dilutions 1:100 and 1:400. Samples were injected for 3min at 10µL/min. Binding responses (from a report point 10s after the end of injection) were fit to a calibration curve using the T200 evaluation software to determine antigen-specific and total IgG concentrations.

Neutralization assay

Serum samples were tested for neutralizing activity in a TZM-bl assay [25] with a pre-bleed pool as negative control. Data are reported as ID₅₀, 50% inhibitory dilution values.

Statistical Analyses

Repeated Measures ANOVA followed by false discovery rate adjustment was used for longitudinal assays. Area under the curve (AUC) was calculated following the trapezoid rule after baseline subtraction. The Kruskal-Wallis test was used for comparison among multiple groups followed by Bonferroni adjustment. For SPR, a Linear Mixed Model, Repeated Measures ANOVA was followed by Tukey-Kramer adjustment. First order autoregressive covariance structure was used to account for within subject correlation. Different comparison adjustment methods and stringent or flexible adjustments were used depending on the number of comparisons. Analyses were performed with SAS V9.3 (SAS Inc).

RESULTS

Co-immunization strategy of rabbits with gp160 DNA and gp140 protein

Five groups of rabbits (n=4 per group) were immunized four times on weeks 0, 4, 12, and 20 with Env (trimeric gp140) protein either alone or in combination with gp160 env DNA (Table 1). Of the five, three groups were co-immunized with plasmid DNA encoding gp160 and gp140 Env protein: (i) subtype B DNA plus subtype B protein (Matched B; SF162 [26]); (ii) subtype A DNA plus subtype A protein (Matched A; QA461e2TAIV [27]); (iii) subtype B DNA plus subtype A protein (Mismatched). As controls, two groups were immunized with subtype B protein: (iv) empty vector DNA plus subtype B protein (Empty Vector); and (v) subtype B protein alone (Protein B). At each immunization, rabbits received 50µg of gp140 in PEI adjuvant and 36 µg of DNA delivered by Gene Gun.

Binding antibody responses are similar in Matched and Mismatched vaccine groups

We evaluated Env-specific binding antibody responses longitudinally by ELISA against trimeric subtype A and B antigens. Strong responses were detected in all groups after two immunizations that were maintained or boosted by subsequent immunizations (Figure 1A). We observed no difference in responses between the Empty Vector and Protein B groups ($P>0.38$), thus showing no adjuvant effect from the vector alone. A similar absence of adjuvant effect by the vector alone was reported previously in a DNA prime-protein boost study [28].

Overall binding potency was determined by calculating the Area Under the Curve (AUC) (Figure 1B). The Matched A and Mismatched groups developed the strongest response against the subtype A antigen compared to controls ($P=0.015$ and $P=0.05$, respectively). As expected, the Matched A group had higher subtype A binding antibodies than the Protein B group ($P=0.05$). Similarly, the Matched B group developed the most potent subtype B-specific binding antibody response, significantly stronger than the Matched A group ($P=0.004$). Subtype A binding responses were indistinguishable between Matched A and Mismatched groups, both of which received subtype A protein.

DNA+protein co-immunizations enhance avidity

We measured antibody avidity to autologous antigens two weeks after immunizations by comparing the binding titers after treatment with 8M urea or PBS (Figure 2). The Mismatched and Matched A groups developed the strongest avidity against the autologous subtype A antigen compared to the Empty Vector group ($P=0.0260$ and $P=0.0569$, respectively) and the Protein B group ($P=0.0160$ and $P=0.0248$, respectively). The Matched B group had a higher avidity towards the autologous B envelope than the Matched A group ($P=0.01$). Not surprisingly, these data also show that the co-immunization vaccine strategies resulted in stronger avidity for their respective cognate subtypes. Both the Empty Vector and the Protein B groups had a significantly higher avidity to the subtype B antigen than the Matched A group ($P=0.0329$ for both). Furthermore, the Matched B group also had a significantly higher avidity to the subtype B antigen than the Mismatched group that was immunized with subtype B DNA and subtype A protein ($P=0.03$). These data suggest that the protein component is the dominant partner for increasing avidity with this combination regimen.

Env-specific antibodies are enriched by DNA+protein co-immunizations

To further evaluate the relative contribution of each vaccine component on antibody production, we used surface plasmon resonance (SPR) to measure the total amount of subtype A- or B-trimeric gp140-specific antibody responses. Since the binding antibody

titers and avidity were indistinguishable between the Empty Vector and the Protein B control groups, we used the Protein B group as control for the SPR analysis. Overall, we found that the antigen-specific responses were nearly identical and significantly higher in the Mismatched and the Matched B groups compared to the protein only group ($P=0.0035$ and $P=0.003$, respectively, Figure 3A).

Consistent with the binding and avidity results, the vaccines with matched subtype components elicited higher antigen-specific responses by SPR against their cognate antigens (Figure 3B), and the Mismatched strategy resulted in comparable levels of antigen-specific responses against both subtype A and B antigens ($P=0.6167$). For example, the Matched A group had significantly higher subtype A antigen-specific responses than the Protein B and the Matched B groups ($P=0.0035$ and $P=0.0421$, respectively), and the Matched B group elicited significantly higher subtype B antigen-specific responses than the Matched A group ($P<0.0001$). Interestingly, the Mismatched vaccine elicited significantly stronger subtype A antigen-specific responses than the Matched B group ($P=0.0063$) and stronger subtype B antigen-specific responses than the Matched A group (unadjusted $P=0.0392$). Finally, we saw no difference in the responses elicited by the Mismatched vaccine and the Matched A vaccine against the subtype A antigen ($P=0.9981$). Taken together, our SPR results show that protein components drive strong cognate antigen-specific responses and mismatching could potentially provide an advantage in cross reactivity.

Co-immunizations increase the rate of NAb development and their potency

We measured neutralization activity against the subtype A and B viruses that were the source of immunogens in this study. Rabbits co-immunized with Mismatched DNA+Protein vaccines developed low subtype A NAb after two immunizations (Figure 4A), and the Mismatched vaccine regimen resulted in higher subtype A NAb than the Protein B and the Empty Vector strategies ($P=0.0375$ and $P=0.0067$, respectively). In contrast, rabbits in all groups developed NAb against the subtype B virus after two immunizations, and subsequent co-immunizations greatly potentiated subtype B NAb in the Matched B and Mismatched groups. The greater dynamic range observed here with clade B SF162 may be due to its high sensitivity to neutralization. The Matched B and Mismatched groups had significantly higher subtype B NAb than the Matched A group ($P=0.0007$ for both), therefore showing that DNA+Protein vaccines elicited higher NAb against their cognate antigens. The Matched B and Mismatched groups had significantly higher subtype B NAb than the Empty Vector group ($P=0.0083$ and $P=0.0405$, respectively) and the Matched B group also had stronger subtype B NAb than the Protein B group ($P=0.0295$) thereby illustrating the influence of the env DNA component. The Empty Vector and the Protein B regimens resulted in higher subtype B NAb than the Matched A group ($P=0.0295$ and $P=0.0083$ respectively), thus showing that the autologous NAb response is mainly driven by the protein component.

We performed AUC analyses to measure the overall potency of NAb (Figure 4B). Co-immunization vaccine strategies resulted in significantly greater potency of autologous NAb. The Mismatched group developed the strongest NAb against the subtype A virus ($P=0.034$ vs Empty Vector), whereas the Matched B group developed the most potent NAb against the subtype B virus ($P=0.010$ vs Matched A). No differences in subtype A or B NAb were detected between the Mismatched and either of the Matched groups.

Effect of DNA+protein co-immunization on neutralization breadth

The model immunogens used in this study have not elicited heterologous NAb with previous vaccine regimens [14, 29, 30]. Considering the improvements in avidity and neutralization potency mediated by the DNA+protein co-immunizations, we tested sera after

the final immunization for neutralization of heterologous viruses (Table 2). Tier 1B, subtype B viruses BaL.26 and SS1196.1 were modestly neutralized by sera from all rabbits in Matched B and Mismatched groups. In addition, 75% of rabbits in the Matched B group neutralized the subtype C virus ZM109F.PB4 at low titers. Matched A Rabbit #1 serum had low level neutralization of all viruses tested, but the Protein B and Matched A groups had two non-responders.

DISCUSSION

There has been progress in developing HIV and SIV vaccines that can elicit strong T cell responses [10], but the components and delivery systems to invoke strong B cell responses are not fully developed [31]. It is therefore critically important to develop immunization strategies that accelerate the humoral response and enhance avidity. Earlier animal studies have shown that avidity was inversely correlated with peak post-challenge viremia [9]. Previously, we reported that co-immunizations using gp160-DNA and a recombinant HIV-Env scaffold protein induced NABs in rabbits and Env-specific CTL in mice. We further showed that boosting in the setting of DNA priming with DNA+gp140 accelerated NAB responses in rabbits [19, 20]. Additionally, it was recently shown that DNA+protein immunization of NHPs conferred neutralization breadth and some protection from SIV challenge [12, 21]. Comparing the antibody response elicited by co-immunizations with DNA expressing model gp160 antigens plus trimeric gp140 protein, DNA vector plus protein or protein alone to determine the relative contribution of each vaccine component is a novel aspect of the current study. Moreover, we used for the first time a novel calibration-free concentration analysis (CFCA) method to assess antigen-specific binding antibody responses in unpurified serum samples. Binding and avidity antibody data showed that the protein component strongly influences the antibody specificity, and the DNA component exerts influence in generating autologous NABs. Mismatching the DNA and protein components resulted in comparable or higher humoral responses than Matched vaccines.

Numerous immunization studies have used Envelope immunogens to elicit NABs in various animal models, and, these Envs have induced fairly weak NABs developing only after multiple immunizations [8, 13, 17, 18, 29, 30, 32–34]. However, DNA vaccines are distinct from conventional vaccines because they stimulate both humoral and cellular responses against antigenic determinants expressed *in vivo* similar to natural exposure to the pathogen; despite their low immunogenicity, they act as intrinsic adjuvants [35]. Thus, use of DNA plasmids in prime-boost regimens is an attractive approach to increase immunogenicity, although this prolongs immunization schedules. In contrast, our DNA+protein co-immunization strategy accelerated the development of binding and neutralizing antibodies compared to vaccination with protein only. Similar results were obtained with DNA+protein co-immunizations in dengue virus and Japanese Encephalitis Virus (JEV) murine vaccine studies [36, 37]. DNA+protein co-immunizations were also successful at eliciting higher binding antibody and T cell responses against hepatitis C [38]. In addition, our results reveal that co-immunization also accelerated the development of HIV Env-specific antibody avidity, thus showing the advantage of using this approach.

The protein component was the driving factor for elicitation of JE-specific NABs when administered as a vaccine mixture with DNA [39] and as a DNA prime–protein boost vaccine [36]. Our findings also show that the protein component of the vaccine has a stronger influence on antibody specificity with higher binding and neutralizing antibody responses against the envelope cognate to the protein component. However, previous studies also showed that DNA priming improves the magnitude and quality of antibody against primary HIV-1 isolates as well as the immunogenicity of the specific Env, which is not accomplished with protein alone [40]. The ability of the DNA component to focus NABs on

conserved regions [28] and enhance avidity against Env protein vaccines [41] may have mediated this effect. Similarly we demonstrate here that the DNA component also contributes to the antibody response, because co-immunizations enhance antibody binding, antibody avidity, and potency of NAb, and accelerate the rate of NAb development.

The DNA+protein combinations elicited higher antigen-specific responses towards their cognate antigens, as demonstrated by binding and neutralizing antibody data, but the Mismatched group had comparable or at least in one case better responses than the Matched groups towards their cognate antigens. Indeed, the Mismatched vaccine displayed strong binding titers against antigens of both subtypes. It also improved subtype A NAb, as shown by the Mismatched group having the highest titers of subtype A NAb, while maintaining strong subtype B NAb. Because this study is one using model antigens that principally target V3 [13, 24]), we did not explore V2 responses, and we can only speculate if the results that we obtained can be generalized for transmitter/founder Envs or other primary Envs. Nonetheless, these results are corroborated by a previous DNA prime–protein boost vaccine study showing that a polyvalent heterologous protein boost elicit a broader NAb response than a homologous boost [41].

In conclusion, our findings show that DNA+protein co-immunization accelerates and enhances binding and NAb responses and that the DNA empty vector component does not contribute. Our results also underscore the role of intrinsic Env immunogenicity in inducing NAb breadth, as despite enhancing the overall antibody response, the effect of DNA+protein co-immunizations using model antigens on NAb breadth was less impressive. Uncleaved gp140 trimers have been shown to be less stable and display aberrant conformations compared to the new cleaved BG505 SOSIP.664 gp140 trimer[42], and thus may also contribute to this effect. The current study begins to address one obstacle to eliciting potent, broad NAb through Env immunizations by shortening the vaccine regimen. We further highlight the importance of considering intrinsic Env immunogenicity in the selection of future immunogens. This co-immunization approach has translational potential for HIV vaccine design when used with newly discovered or engineered Env immunogens.

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Highlights for Review

We co-immunized rabbits with clade A or B HIV gp160 plasmid DNA plus Env gp140 trimer

DNA+Protein co-immunization was superior to immunization with protein alone

Co-immunization enhanced antibody kinetics, magnitude, avidity, neutralizing potency

The protein component drove the antibody avidity and neutralizing responses

Mismatched vaccines elicited comparable/better humoral responses than matched ones

Figure 1. Autologous Envelope-binding antibody response

(A) Longitudinal analysis of binding antibody titers measured by kinetic ELISA against autologous (vaccine) subtype A (Q461e2TAIV, left) and B (SF162, right) trimeric gp140. Arrows indicate co-immunization timepoints. (B) Area Under the Curve analysis of longitudinal binding curves, expressed as relative units. Each symbol represents an individual rabbit. *P* values are indicated (Kruskal-Wallis test followed by Bonferroni adjustment).

Figure 2. Potency of antibody avidity to autologous Envs

Avidity indices were determined by 8M urea displacement ELISA two weeks after immunization against subtype A (Q461e2TAIV, left) and B (SF162, right) vaccine gp140 Envs. *P* values were determined by Repeated Measures ANOVA followed by false discovery rate adjustment. For autologous subtype A avidity indices: Mismatched vs Empty Vector, $P=0.0260$; Matched A vs Empty Vector, $P=0.0569$; Matched A vs Protein B, $P=0.0248$ and Mismatched vs Protein B, $P=0.0160$. For autologous subtype B avidity indices: Matched B vs Matched A, $P=0.01$; Matched B vs Mismatched, $P=0.03$; Empty Vector vs Matched A, $P=0.0329$ and Protein B vs Matched A, $P=0.0329$.

A. **B.** **Figure 3. Subtype A and B autologous Envelope-specific antibodies**

Subtype A (Q461e2TAIV) and B (SF162) Envelope-specific antibodies present in rabbit antisera two weeks after immunization were assessed by surface plasmon resonance and reported as percent of total IgG. (A) Total Subtype A and B Envelope-specific IgG responses in each vaccine group. (B) Subtype-specific Envelope IgG response (Subtype A Q461e2TAIV, closed bars; Subtype B SF162, open bars) within each vaccine group. P values are indicated (Linear Mixed Model Repeated Measures ANOVA with Tukey-Kramer adjustment).

Figure 4. Neutralization potency against vaccine antigens

Rabbit antisera were tested for neutralization of autologous subtype A (Q461e2TAIV, left panels) and B (SF162, right panels) viruses by TZM-bl neutralization assay. **(A)** 50% neutralization (ID₅₀) of rabbit immune sera displayed longitudinally. Arrows indicate co-immunization timepoints. *P* values were determined by Repeated Measures ANOVA followed by false discovery rate adjustment. For autologous subtype A NAb: Mismatched vs Protein B, $P=0.0375$; Mismatched vs Empty Vector, $P=0.0067$. For autologous subtype B NAb: Matched B vs Matched A, $P=0.0007$; Mismatched vs Matched A, $P=0.0007$; Matched B vs Empty Vector, $P=0.0083$; Mismatched vs Empty Vector, $P=0.0405$; Matched B vs Protein B, $P=0.0295$; Empty Vector vs Matched A, $P=0.0295$ and Protein B vs Matched A, $P=0.0083$. **(B)** Area Under the Curve analysis of longitudinal neutralization data, expressed as relative units. Each symbol represents an individual rabbit. *P* values are indicated (Kruskal-Wallis test followed by Bonferroni adjustment).

Table 1

Co-immunization strategies

Vaccine	DNA component	Protein component
Protein B	None	Subtype B (SF162)
Empty Vector/Protein B	pEMC*	Subtype B (SF162)
Matched B	Subtype B (SF162)	Subtype B (SF162)
Mismatched	Subtype B (SF162)	Subtype A (Q461e2TAIV)
Matched A	Subtype A (Q461e2TAIV)	Subtype A (Q461e2TAIV)

Five groups of rabbits (n=4) were co-immunized with different combinations of gp160 envelope DNA (36 µg via Gene gun, intradermal) and gp140 trimeric protein (50 µg, intramuscular) in presence of PEI adjuvant. Rabbits were vaccinated at weeks 0, 4, 12 and 20.

Table 2

Heterologous neutralization activity of rabbit immune sera

Vaccine groups	Viral isolates						
	BaL.26 (B)	SSI196.1 (B)	JRCSE (B)	YU-2 (B)	QH0692 (B)	TRO.11 (B)	ZM109F.PB4 (C)
Protein B	27	15					10
	23	14					13
	27	15					
Matched B	71	57					31
	30	30					17
	34	37					21
	49	73	18				
	20	25					
Mismatched	21	11					
	24	41					
	58	78	62	49	59	42	141
Matched A	19	22					19

Serum ID ₅₀
<10
10–20
21–50
51–100
>100