Most rhesus macaques infected with the CCR5-tropic SHIV<sub>AD8</sub> generate cross-reactive antibodies that neutralize multiple HIV-1 strains

Masashi Shingai<sup>a,1</sup>, Olivia K. Donau<sup>a,1</sup>, Stephen D. Schmidt<sup>b,1</sup>, Rajeev Gautam<sup>c</sup>, Ronald J. Plishka<sup>a</sup>, Alicia Buckler-White<sup>a</sup>, Reza Sadjadjour<sup>d</sup>, Wendy R. Lee<sup>e</sup>, Celia C. LaBranche<sup>e</sup>, David C. Montefiori<sup>f</sup>, John R. Mascola<sup>b</sup>, Yoshiaki Nishimura<sup>a</sup>, and Malcolm A. Martin<sup>a,2</sup>

<sup>a</sup>Laboratory of Molecular Microbiology, <sup>b</sup>Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and <sup>c</sup>Department of Surgery, Duke University Medical Center, Durham, NC 27710

Contributed by Malcolm A. Martin, October 8, 2012 (sent for review September 5, 2012)

The induction of broadly reacting neutralizing antibodies has been a major goal of HIV vaccine research. Characterization of a pathogenic CCR5 (R5)-tropic SIV/HIV chimeric virus (SHIV) molecular clone (SHIV<sub>AD8</sub>) revealed that eight of eight infected animals developed cross-reactive neutralizing antibodies (NAbs) directed against an envelope glycoprotein derived from the heterologous HIV-1<sub>1073</sub> strain. A panel of plasmas, collected from monkeys inoculated with either molecularly cloned or uncloned SHIV<sub>AD8</sub> stocks, exhibited cross-neutralization against multiple tier 1 and tier 2 HIV-1 clade B isolates. One SHIV<sub>AD8</sub>-infected animal also developed NAbs against clades A and C HIV-1 strains. In this particular infected macaque, the cross-reacting anti-HIV-1 NAbs produced between weeks 7 and 13 were directed against a neutralization-sensitive virus strain, whereas neutralizing activities emerging at weeks 41–51 targeted more neutralization-resistant HIV-1 isolates. These results indicate that the SHIV<sub>AD8</sub> macaque model represents a potentially valuable experimental system for investigating B-cell maturation and the induction of cross-reactive NAbs directed against multiple HIV-1 strains.

A major challenge in HIV vaccine research has been the development of immunogens capable of eliciting potent, broadly acting, neutralizing antibodies (NAbs). It is now appreciated that 10–30% of HIV-1–infected individuals produce cross-reactive NAbs of significant breadth (1–6). Less than 1% of such persons, so-called “elite neutralizers,” produce potent cross-clade-neutralizing activity, but only 2–3 y after virus acquisition (6). Although the emergence of broadly reacting anti-HIV-1 NAbs in elite neutralizers has been associated with multiple rounds of somatic hypermutation (7), little is known about vaccine strategies able to elicit such antibodies. Longitudinal studies of HIV-1–infected persons have suggested that set-point plasma virus loads, CD4<sup>+</sup> T-cell levels, duration of the infection, or antibody-binding avidity may contribute to the development of cross-reacting NAbs (5, 8, 9). It is also possible that the induction of such antibodies depends on unique gpl20 epitopes associated with specific HIV-1 strains and individual B-cell repertoires or is simply a random process (10, 11). A nonhuman primate model capable of generating cross-reactive anti-HIV-1-neutralizing activity could provide answers to some of these questions and contribute to the development of an effective prophylactic vaccine. In this regard, we recently reported that one rhesus monkey, inoculated with an uncloned preparation of the R5-tropic SHIV<sub>AD8</sub> developed broad, potent, and glycan-specific NAbs with cross-reactive activity against virus isolates from different HIV-1 clades similar to that described for “elite” HIV-1 neutralizers (12).

In this study, we describe the construction of a pathogenic SHIV<sub>AD8</sub> molecular clone (SHIV<sub>AD8-EO</sub>). During its characterization, we discovered that eight of eight SHIV<sub>AD8-EO</sub>-infected animals generated cross-reactive NAbs directed against a SHIV carrying an envelope glycoprotein derived from a different HIV-1 isolate (namely HIV-1<sub>1073</sub>). Because this result suggested that a majority of monkeys infected with the SHIV<sub>AD8-EO</sub> family of viruses might be able to generate NAbs against heterologous HIV-1 isolates, plasma samples collected from a cohort of 11 rhesus macaques, infected with either uncloned SHIV<sub>AD8</sub> or SHIV<sub>AD8-EO</sub> molecular clones, were tested for their capacity to neutralize a panel of clade A, B, and C HIV-1 isolates. All of the plasmas from this group of 11 animals neutralized several tier 1A and 1B clade B HIV-1 isolates. Three of the 11 macaques also generated >1:100 IC<sub>50</sub> neutralization titers against some tier 2 clade B HIV-1 isolates, and one of the three produced significant NAbs titers against some clade A and C isolates. Taken together, these findings indicate that SHIV<sub>AD8</sub> is uniquely immunogenic during infections of rhesus monkeys and may be a particularly useful reagent for identifying viral determinants that drive B-cell maturation resulting in cross-reactive anti-HIV-1 NAbs.

Results

Infection of Rhesus Monkeys with the SHIV<sub>AD8-EO</sub> Molecular Clone.

Although we had previously reported the construction and characterization of the R5-tropic SHIV<sub>AD8</sub> and had prepared uncloned SHIV<sub>AD8</sub> vaccine stocks and SHIV<sub>AD8-EO</sub> molecular clones, were tested for their capacity to neutralize a panel of clade A, B, and C HIV-1 isolates. All of the plasmas from this group of 11 animals neutralized several tier 1A and 1B clade B HIV-1 isolates. Three of the 11 macaques also generated >1:100 IC<sub>50</sub> neutralization titers against some tier 2 clade B HIV-1 isolates, and one of the three produced significant NAbs titers against some clade A and C isolates. Taken together, these findings indicate that SHIV<sub>AD8</sub> is uniquely immunogenic during infections of rhesus monkeys and may be a particularly useful reagent for identifying viral determinants that drive B-cell maturation resulting in cross-reactive anti-HIV-1 NAbs.


The authors declare no conflict of interest.

1M.S., O.K.D., and S.D.S. contributed equally to this article.
2To whom correspondence should be addressed. E-mail: mmartin@niaid.nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217443109/-/DCSupplemental.
was accompanied by marked weight loss. Macaque DC6W also experienced marked weight loss and was euthanized at week 111 with a gastric lymphoma. Macaque CD8T was euthanized at week 117 with multiple intra-abdominal lymphomas.

Neutralizing Antibodies Generated in SHIVAD8-Infected Macaques. We recently reported that only 3 of 19 macaques inoculated with SHIVAD8 viruses developed sustained levels of autologous Nabs (13). In the current study, autologous Nabs, developing in eight of the macaques infected with the SHIVAD8EO molecular clone were initially assessed using plasma samples diluted 1:20. As shown in Fig. 2, five of the eight animals produced autologous neutralizing activity (>50% neutralization). In four of these macaques (DC6W, DCF1, FZH, and JG7), autologous Nabs became measurable between weeks 20 and 40 PI, but were delayed until week 74 in the fifth (DC8T) animal. Individual samples from three of these monkeys (DC6W, DCF1, and JG7) had autologous neutralization IC50 titers ranging from 1:148 to 1:161.

In an earlier study, we reported that one rhesus monkey inoculated with uncloned SHIVAD8 developed extraordinarily broad, cross-clade, and high-titered Nabs similar to that described for HIV-1 “elite neutralizers” (12). This potent neutralizing activity targeted the gp120 N332 glycan. Because we were curious whether animals infected with the SHIVAD8-EO molecular clone might also produce antibodies able to neutralize a heterologous HIV-1 isolate, plasma samples (1:20 dilution) from the same eight SHIVAD8-EO–infected monkeys were tested for their capacity to neutralize pseudovirions bearing the CXCR4 (X4)-tropic SHIVDH12-CL7 envelope glycoprotein, originally derived from the HIV-1DH12 isolate (15–17). The HIV-1–derived env genes present in SHIVAD8-EO and SHIVDH12-CL7 are 90% and 84% identical at the level of nucleotide and amino acid sequences, respectively. As shown in Fig. 2B, all eight plasmas, including samples from animals (DC0L, DC7W, and DCV9) with no or extremely low autologous Nabs, exhibited neutralizing activity against SHIVDH12.

We next determined the cross-reactive IC50 NAb titers directed against SHIVDH12 present in the plasmas of a different cohort of 11 macaques, which had been inoculated with molecularly cloned SHIVAD8 or recently described SHIVAD8 swarm stocks (13) (Fig. 3A). Only 4 (macaques DC6W, DCF1, DA55, DA70) of these 11 SHIVAD8–infected animals had developed autologous Nabs. Nonetheless, the plasmas from all 11 SHIVAD8–infected animals generated anti-SHIVDH12 neutralizing IC50 titers (ranging from 1:236 to 1:3,040).

The production of cross-reacting NAbs against SHIVDH12 by SHIVAD8–infected monkeys raised the possibility that a similar activity might have also been generated against other HIV-1
isolates. Plasma samples from the same cohort of 11 animals, infected with the molecularly cloned or uncloned SHIV\textsubscript{AD8} viruses, were assessed for their capacity to neutralize a panel of clade A, B, and C HIV-1 strains. As shown in Fig. 3\textbf{B} and \textbf{C}, plasmas from all of the monkeys possessed neutralizing activity against tier 1A (very high sensitivity) and tier 1B (above-average sensitivity) clade B HIV-1 isolates (18). In addition, three of the macaques (CL5E, DCF1, and DA55) generated >1:100 IC\textsubscript{50} NAb titers against some tier 2 (moderate sensitivity) clade B HIV-1 strains (HIV-1\textsubscript{JR-FL}, HIV-1\textsubscript{76515}, HIV-1\textsubscript{TRO.11}, and HIV-1\textsubscript{CAAN.A2}). The plasma from monkey CL5E exhibited the widest breadth, including neutralization activity against clade A and C HIV-1 strains.

A neutralization profile for the previously reported (12) "elite neutralizer" macaque CE8J at the time of its euthanasia at week 100 PI is also shown at the bottom of each panel. Value between 20 and 99, green; value between 100 and 999, yellow; value ≥1,000, red. Blank cell indicates that value was not determined.

Shingai et al. PNAS Early Edition | 3 of 6

**Fig. 3.** Macaques infected with uncloned or molecularly cloned SHIV\textsubscript{AD8} viruses develop cross-reacting NAbs against multiple HIV-1 strains. IC\textsubscript{50} neutralization titers against the highly sensitive SHIV\textsubscript{DH12} (\textbf{A}), the indicated clade B (\textbf{B}) and clade A and C (\textbf{C}) HIV-1 isolates in plasmas of a cohort of 11 SHIV\textsubscript{AD8}-infected animals were determined by reciprocal dilution and assay in TZM-bl cells. Values represent reciprocal serum dilution required to achieve 50% neutralization. The previously reported (12) neutralizing activity of week 100 plasma from animal CE8J is shown at the bottom of each panel. Value between 20 and 99, green; value between 100 and 999, yellow; value ≥1,000, red. Blank cell indicates that value was not determined.

**Table S1.**

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Weeks post inoculation</th>
<th>Tier</th>
<th>Clade B</th>
<th>Clade C</th>
<th>Non-HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE8J</td>
<td>100 SHIV\textsubscript{AD8}</td>
<td>1,111</td>
<td>120</td>
<td>&lt;5</td>
<td>144</td>
</tr>
</tbody>
</table>
high-titered antibodies against the tier 1B SS1196.1 HIV-1 isolate (Figs. 3 and 5), all had set-point viral loads between 10^3 and 10^4 viral RNA copies/mL, whereas animal JLD, with a lower level (~200 RNA copies/mL) of plasma viremia (Fig. 5), had a very low neutralization titer against the SS1196.1 strain. All three macaques (CL5E, DCF1, and DA55) producing NAbs against the more resistant tier 2 HIV-1 isolates had set-point plasma viremia levels between 10^5 and 10^6 RNA copies/mL.

The delayed appearance of phase 2 neutralization activity in macaque CL5E is also reminiscent of the emergence of the previously described potent cross-clade NAbs generated by SHIV AD8–infected monkey CEF8, which first became detectable between weeks 32 and 36 PI (12). It is worth noting that a similar two-phase pattern of cross-reacting NAbs development has been reported in a longitudinal study of HIV-1–infected individuals (25). The emergence of the second phase required ~2.5 y during these HIV-1 infections whereas SHIV AD8–infected animals developed activity against more difficult-to-neutralize HIV-1 strains within 1 y of virus inoculation.

Although a time course of NAb development was not determined for each of the 11 SHIV AD8–infected animals listed in Fig. 3, it is quite likely that, in addition to monkey CL5E, macaques DCF1 and DA55, both of which generated neutralization IC_{50} titers >1:100 against the relatively resistant tier 2 HIV-1 JR-FL isolate, also produced both early and late-phase NAbs. Thus, of the 11 SHIV AD8–infected animals intensively evaluated for the development of cross-reacting NAbs (Fig. 3), 3 monkeys likely exhibited a “two-phase” phenotype of NAb development, and 8 monkeys were able to neutralize only sensitive HIV-1 strains. It is not currently known whether or not a common epitope is targeted by the early emerging cross-reactive NAbs in the latter group of SHIV AD8–infected animals.

At present, we do not know which epitopes associated with the SHIV AD8 envelope glycoprotein are driving the production of cross-reactive neutralizing activity or which epitopes in heterologous HIV-1 strains are targeted for neutralization. Because a majority of SHIV AD8–infected monkeys are able to generate cross-reactive neutralizing activity against multiple HIV-1 isolates, studies of B-cell maturation and the generation of broadly reacting NAbs, triggered by a single HIV-1 envelope glycoprotein (namely HIV-1 AD8) are now possible using the macaque model. One could envisage experiments monitoring B-cell maturation patterns and NAb development in the three SHIV AD8–infected monkeys (CL5E, DCF1, and DA55 in Fig. 3) able to neutralize the tier 2 HIV-1 JR-FL isolate at IC_{50} titers >1:100. The results obtained from such a study could guide the development of immunogens designed to elicit late-phase cross-reactive anti–HIV-1 NAbs.
The failure of many SHIVAD8-infected animals to generate potent and sustained autologous NAbs remains an enigma. We recently reported that only 3 of 19 monkeys inoculated with SHIVAD8 swarm stocks produced autologous neutralizing activity (13), and in the present study only 5 of 8 macaques inoculated with the SHIVAD8-EO molecular clone developed autologous NAbs (Fig. 2A). Perhaps envelope glycoproteins associated with relatively neutralization-resistant HIV-1 strains like the tier 3 HIV-1AD8, rather than envelopes from tissue-culture-adapted virus isolates, may present unique epitopes to the immune system, which elicit an ensemble of cross-reactive NAbs that emerge at different times and possess varying potencies.

Materials and Methods

Construction and Characterization of a Pathogenic SHIVAD8 Molecular Clone. The strategy used to obtain a potentially pathogenic SHIVAD8 molecular clone was (i) to amplify env gene-containing segments from a previously described (14) cohort of nine animals, infected with serially passaged SHIVAD8 derivatives (Table S1); (ii) to construct full-length SHIV molecular clones carrying some of these amplified env genes; and (iii) to assess the infectivity of reconstructed viruses in rhesus PBMC and in inoculated macaques. The detailed construction of SHIVAD8 molecular clones and the characterization of resulting virus stocks are described in SI Materials and Methods.

Cell Culture. HEK293T (293T) and TZM-bl cells were cultured in Dulbecco’s modified Eagle’s MEM supplemented with 10% (vol/vol) heat-inactivated FBS. Rhesus monkey PBMCs were prepared and cultured as described previously (26).

Animal Experiments. Rhesus macaques (Macaca mulatta) were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (27) and were housed in a biosafety level 2 NIAID facility. Phlebotomies, euthanasia, and tissue sample collections were performed as previously described (28). BAL fluid lymphocytes were prepared as previously described (29). All animals were negative for the MHC class I Mamu-A*01 allele.
Viral RNA levels in plasma were 32). T8526. exhibited a tier 3 phenotype in this assay. SHIV 769. 13052. The neutralization activity present in plasma samples 1636. Proc Natl Acad Sci USA 81(12):6402 74(15):6935 25(8):1398 2055. 181. 46(6):1896. We thank Keiko Tomioka, Robin Kruthers, and Ranjini lycenosis activities against subtype B HIV-1 isolates (37).

Neutralization Phenotypes of HIV-1 and SHIV Preparations. Using standardized plasma pools from HIV-1–infected individuals (18), we determined that the molecularly cloned SHIVAD8-EGO exhibited a tier 2 neutralization phenotype (Table S1), the level usually associated with circulating HIV-1 strains. The parental HIV-1AD8 exhibited a tier 3 phenotype in this assay. SHIVDT2LCL displayed a tier 2 neutralization phenotype with this pool of plasma.

ACKNOWLEDGMENTS. We thank Keiko Tomioka, Robin Kruthers, and Ranjini lycenosis activities against subtype B HIV-1 isolates (37).

Neutralization Phenotypes of HIV-1 and SHIV Preparations. Using standardized plasma pools from HIV-1–infected individuals (18), we determined that the molecularly cloned SHIVAD8-EGO exhibited a tier 2 neutralization phenotype (Table S1), the level usually associated with circulating HIV-1 strains. The parental HIV-1AD8 exhibited a tier 3 phenotype in this assay. SHIVDT2LCL displayed a tier 2 neutralization phenotype with this pool of plasma.

ACKNOWLEDGMENTS. We thank Keiko Tomioka, Robin Kruthers, and Ranjini lycenosis activities against subtype B HIV-1 isolates (37).

Neutralization Phenotypes of HIV-1 and SHIV Preparations. Using standardized plasma pools from HIV-1–infected individuals (18), we determined that the molecularly cloned SHIVAD8-EGO exhibited a tier 2 neutralization phenotype (Table S1), the level usually associated with circulating HIV-1 strains. The parental HIV-1AD8 exhibited a tier 3 phenotype in this assay. SHIVDT2LCL displayed a tier 2 neutralization phenotype with this pool of plasma.

ACKNOWLEDGMENTS. We thank Keiko Tomioka, Robin Kruthers, and Ranjini lycenosis activities against subtype B HIV-1 isolates (37).

Neutralization Phenotypes of HIV-1 and SHIV Preparations. Using standardized plasma pools from HIV-1–infected individuals (18), we determined that the molecularly cloned SHIVAD8-EGO exhibited a tier 2 neutralization phenotype (Table S1), the level usually associated with circulating HIV-1 strains. The parental HIV-1AD8 exhibited a tier 3 phenotype in this assay. SHIVDT2LCL displayed a tier 2 neutralization phenotype with this pool of plasma.

ACKNOWLEDGMENTS. We thank Keiko Tomioka, Robin Kruthers, and Ranjini lycenosis activities against subtype B HIV-1 isolates (37).

Neutralization Phenotypes of HIV-1 and SHIV Preparations. Using standardized plasma pools from HIV-1–infected individuals (18), we determined that the molecularly cloned SHIVAD8-EGO exhibited a tier 2 neutralization phenotype (Table S1), the level usually associated with circulating HIV-1 strains. The parental HIV-1AD8 exhibited a tier 3 phenotype in this assay. SHIVDT2LCL displayed a tier 2 neutralization phenotype with this pool of plasma.

ACKNOWLEDGMENTS. We thank Keiko Tomioka, Robin Kruthers, and Ranjini lycenosis activities against subtype B HIV-1 isolates (37).

Neutralization Phenotypes of HIV-1 and SHIV Preparations. Using standardized plasma pools from HIV-1–infected individuals (18), we determined that the molecularly cloned SHIVAD8-EGO exhibited a tier 2 neutralization phenotype (Table S1), the level usually associated with circulating HIV-1 strains. The parental HIV-1AD8 exhibited a tier 3 phenotype in this assay. SHIVDT2LCL displayed a tier 2 neutralization phenotype with this pool of plasma.