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Recombinant Canarypox Vaccine-Elicited CTL Specific for Dominant and Subdominant Simian Immunodeficiency Virus Epitopes in Rhesus Monkeys

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Since virus-specific CTL play a central role in containing HIV replication, a candidate AIDS vaccine should generate virus-specific CTL responses. In this study, the ability of a recombinant canarypox virus expressing SIV Gag-Pol-Env (ALVAC/SIV gag-pol-env) was assessed for its ability to elicit both dominant and subdominant epitope-specific CTL responses in rhesus monkeys. Following a series of five immunizations, memory CTL responses specific for a dominant Gag epitope could be demonstrated in the peripheral blood of vaccinated monkeys. Memory CTL responses to a subdominant Pol epitope were undetectable in these animals. Following challenge with SIVmac251, the experimentally vaccinated animals developed high frequency CTL responses specific for the dominant Gag epitope that emerged in temporal association with the early containment of viral replication. Interestingly, the experimentally vaccinated, but not the control vaccinated animals, developed CTL responses to the subdominant Pol epitope that were detectable only after containment of early viremia. Thus, recombinant canarypox vaccination elicited low frequency, but durable memory CTL populations. The temporal association of the emergence of the dominant epitope-specific response with early viral containment following challenge suggests that this immune response played a role in the accelerated clearing of early viremia in these animals. The later emerging CTL response specific for the subdominant epitope may contribute to the control of viral replication in the setting of chronic infection. The Journal of Immunology, 2002, 168: 1847–1853.

The CD8\(^+\) CTL responses play a critical role in controlling both HIV-1 and SIV replication. During primary infection, containment of HIV-1 and SIV replication is associated with the emergence of virus-specific CTL (1–3). In addition, persistent, potent CTL responses are associated with low virus loads and delayed progression of clinical disease in infected individuals (4–6). Furthermore, CD8\(^+\) T lymphocyte-depleted rhesus monkeys are unable to contain SIV replication during primary or chronic infection (7, 8). Recent studies also indicate that SIV replication following viral challenge is better contained in monkeys with vaccine-elicited virus-specific CTL as compared with control monkeys (9, 10). HIV-1 vaccine candidates should therefore elicit potent virus-specific CTL responses.

A number of novel vaccine approaches are currently being explored to elicit HIV-1-specific CTL (11). These approaches include the use of plasmid DNA and live recombinant vectors. Perhaps the most intensively studied of the live vectors is the canarypox virus (12–14). This pox vector undergoes an abortive replication cycle in nonhuman primates and humans, but it expresses sufficient intracellular protein to elicit T cell immunity (15). Although recombinant canarypox vaccine constructs have undergone extensive early phase testing in human volunteers, their efficiency in eliciting HIV-1-specific CTL responses remains poorly defined (16–20). In light of the data from recent nonhuman primate studies indicating that vaccine-elicited CTL confer protection against AIDS virus spread and disease progression (9, 21, 22), it will be important to learn as much as possible about the ability of recombinant canarypox constructs to elicit CTL responses. Such data will inform in an important way the decision as to whether to proceed with extended efficacy trials of rHIV-1 canarypox constructs in human populations.

The SIV/macaque model has proven to be a powerful system for exploring the immunogenicity of a variety of HIV-1 vaccine prototypes. The utility of nonhuman primates for assessing HIV-1 vaccine immunogenicity has been increased by the definition of CTL epitopes as well as the application of MHC class I/peptide tetramer staining of epitope-specific T lymphocytes (23–25). In particular, an understanding of specific MHC class I molecules of Indian-origin rhesus monkeys and the CTL epitope peptides they present to CD8\(^+\) T lymphocytes has facilitated the quantitative monitoring of CTL populations specific for multiple viral epitopes (26). The present study evaluates the ability of recombinant canarypox vectors to elicit dominant and subdominant SIV epitope-specific CTL responses in rhesus monkeys.

Materials and Methods

Construction of recombinant canarypox vectors

ALVAC/SIV gag-pol-env or vcp180 (27) expresses the SIV env expression cassette under the control of the H\(_6\) promoter, and the SIV\(_{mac251}\) gag-pol

Abbreviations used in this paper: ALVAC, recombinant canarypox virus; B-LCL, B lymphoblastoid cell line.
FIGURE 1. SIV Gag p11C-tetramer binding to CD8+ lymphocytes in the whole blood of the control and ALVAC/SIV gag-pol-env-immunized Mamu-A*01† rhesus monkeys after a fifth immunization. Whole blood from the vaccinated and control monkeys was directly stained with tetramer and mAbs and then lysed, washed, fixed, and analyzed by flow cytometry. The percentage of p11C-tetramer binding represents p11C tetramer-binding CD8αβ+ T cells in unstimulated whole blood.

tetramer staining

Soluble tetrameric Mamu-A*01/p11C and Mamu-A*01-p68A complexes were prepared as described elsewhere (24, 26). PE-labeled tetrameric Mamu-A*01/p11C or Mamu-A*01-p68A complexes (0.2 μg) in conjunction with FITC-labeled anti-human CD8α (Leu2a; BD Biosciences, San Diego, CA), energy-coupled dye-labeled anti-human CD3ε (2ST8-5H7; Beckman Coulter, Fullerton, CA), and APC-labeled anti-rhesus CD3 (FN18) mAbs were used to stain p11C- or p68A-specific CD8+ T cells, as described previously (24, 26). One hundred microliters of whole blood from both vaccinated and control monkeys was directly stained with these reagents, lysed on an Immunoprep Reagent Q-Prep Workstation (Coulter, Fullerton, CA), washed in 3 ml of PBS, and fixed in 0.5 ml of PBS containing 1.5% paraformaldehyde. Alternatively, PBL from rhesus monkeys were isolated and washed in HBSS containing 2% FCS. PBL (5 x 10⁶) were cultured in vitro with p11C for 12 days.

FIGURE 2. ALVAC/SIV gag-pol-env-elicited Gag epitope-specific memory CTL in PBL of the vaccinated monkeys. PBL of the control and ALVAC/SIV gag-pol-env-vaccinated monkeys were cultured in vitro with p11C for 12 days. A. Cells were stained with tetrameric Mamu-A*01/peptide complex. The percentage of p11C-tetramer binding represents p11C tetramer-binding CD8αβ+ T cells. B. p11C peptide-stimulated lymphocytes were also assessed as effector cells in a cytotoxicity assay using as target cells ⁵¹Cr-labeled autologous B-LCL pulsed with peptide p11C. Data represent percent specific lysis of target cells incubated with p11C peptide minus lysis of target cells incubated with the control p11B (ALSEGCTPYDIN) peptide at an E:T ratio of 5:1.
of PBS and were stained with 0.2 μg of PE-labeled tetrameric Mamu-A*01/p11C or Mamu-A*01/p68A complexes in conjunction with FITC-labeled anti-human CD8α (Leu2a; BD Biosciences), energy-coupled dye-labeled anti-human CD8αβ (2ST8-5H7; Beckman Coulter), and APC-labeled anti-human CD3 (FN18) mAb. Then the samples were washed in 3 ml of PBS containing 2% FBS and fixed in 0.5 ml of PBS containing 1.5% paraformaldehyde. Samples were analyzed by four-color flow cytometry on a Coulter EPICS Elite ESP system. Gated CD3+CD8αβ+ T cells were examined for staining with tetrameric Mamu-A*01/p11C or Mamu-A*01/p68A complexes.

**CTL assays**

PBL from rhesus monkeys were isolated and washed in HBSS containing 2% FCS. PBL (5 × 10⁶) in 2 ml of RPMI 1640 medium containing 12% FCS (R12) were cultured in the presence of 1 μg/ml p11C, p68A, or p11B control peptide (ALSEGCTPYDIN) and labeled overnight with 100 μCi/ml ⁵¹Cr were used as targets. After a 4-h incubation at 37°C, supernatants were harvested, mixed with scintillation fluid, and measured by using a Wallac 1450 Microbeta liquid scintillation counter. To measure spontaneous release of ⁵¹Cr, target cells were incubated with 100 μl of 2% Triton X-100. Percent lysis was calculated as: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

**Viral load assay**

Viral load in the plasma of the animals was assessed using a nucleic acid amplification assay (NASBA) for quantifying SIV RNA (31).

**Results**

To assess the immunogenicity of recombinant canarypox vaccines, rhesus monkeys received a series of five inoculations of recombinant vcp180-ALVAC/SIV gag-pol-env or control ALVAC constructs at 0, 1, 6, 12, and 33 mo. A subset of those animals was shown to express the MHC class I allele Mamu-A*01, as determined by PCR-based typing and gene sequencing (32). Therefore, these monkeys could be evaluated for the generation of CTL specific for a number of well-defined SIV epitopes (26). PBL from the Mamu-A*01-restricted dominant Gag epitope p11C and the subdominant Pol epitope p68A. These studies were done by Mamu-A*01/p11C and Mamu-A*01/p68A tetramer staining of unstimulated peripheral blood CD8⁺ T lymphocytes (24). Low level Mamu-A*01/p11C tetramer binding of peripheral blood CD8⁺ T lymphocytes could be demonstrated before and both 2 and 4 wk following the fifth vaccination for CTL specific for the Mamu-A*01-restricted dominant Gag epitope p11C and the subdominant Pol epitope p68A. Although this observation suggested that recombinant canarypox vaccines did not elicit high frequency CTL responses, it did not address the possibility that this series of immunizations might have elicited low frequency, but durable memory CTL responses that could be expanded on exposure of lymphocytes to SIV Ag. To assess this possibility, PBL of these same monkeys were stimulated in vitro with SIV Gag p11C and SIV Pol p68A, and those cells were assessed for p11C and p68A tetramer binding and functional cytotoxicity using standard chromium release assays. In vitro stimulation of PBL from the vaccinated monkeys with p11C peptide expanded the tetramer-positive cell population from 12.4 to 54.5% of CD8⁺ T lymphocytes (Fig. 2A). Gag peptide
p11C-specific functional CTL were readily detected in peptide-stimulated PBL in both the preboost and postboost samples (Fig. 2B). In vitro stimulation of PBL with p68A peptide resulted in the expansion of a small population of the tetramer-positive CD8^+ T lymphocytes (Fig. 3A). Very weak SIV Pol p68A-specific functional CTL responses were detected in the majority of vaccinated animals before the last immunization (Fig. 3B). Those weak responses did not increase following immunization. Thus, this series of recombinant canarypox immunizations elicited a memory CTL response specific for the dominant Gag p11C epitope, but not clearly for the subdominant Pol epitope. However, there was no evidence that the final immunization increased the size of this pool of memory CTL.

The cohort of vaccinated monkeys was then challenged by intrarectal inoculation with 30 monkey infectious dose 50 cell-free SIVmac251 (33) and assessed thereafter for Mamu-A*01/p11C and Mamu-A*01/p68A tetramer-binding CD8^+ T lymphocytes in the peripheral blood. Following viral challenge, we expected to detect an anamnestic CTL response in monkeys with a vaccine-elicited memory CD8^+ CTL response (10, 32). Thus, we expected the virus-specific CTL response to emerge earlier after virus infection or expand to a greater magnitude in the experimentally vaccinated compared with the control vaccinated monkeys. In fact, a higher SIV Gag p11C tetramer-positive cell response was seen during primary infection in the experimentally vaccinated than in the control vaccinated monkeys, with a mean value of 7.3% p11C tetramer-binding CD8^+ T lymphocytes in the peripheral blood of the experimentally immunized monkeys compared with 1.6% in the control immunized monkeys on day 17 postchallenge (Fig. 4A). Application of the two-sided Wilcoxon rank sum test to the tetramer data on day 17 postchallenge yielded a p value of 0.03. These differences between groups, therefore, are statistically significant.

Interestingly, a striking effect of recombinant canarypox vaccination was also seen in the CTL response to the subdominant SIV Pol p68A epitope following viral challenge. The emergence of CTL specific for p68A was detected later than CTL for p11C, on day 42 compared with day 17. Interestingly, this Pol-specific response could be detected in the peripheral blood of the experimentally vaccinated, but not the control vaccinated monkeys (Fig. 4B). The magnitude of the response to this subdominant epitope was lower than that directed to the dominant SIV Gag epitope, with a median response of 5.15% p11C as compared with 0.25% p68A-binding CD8^+ T lymphocytes on day 42 following challenge (Fig. 4).

These vaccine-elicited CD8^+ T cell responses did not provide protection against viral infection. Although a difference was observed in the time to viremia control in vaccinated monkeys, this difference did not achieve statistical significance (33). However, a difference in plasma viral loads between these two groups of monkeys during chronic infection would have been difficult to discern.
since all of these *Mamu-A*01 rhesus monkeys had undetectable plasma viral RNA following primary viremia. A significant difference in control of viremia was observed in *Mamu A*01 macaques in comparing the experimentally and control vaccinated animals (33).

A close temporal relationship was observed between the peak SIV Gag p11C tetramer-positive CD8<sup>+</sup> T cell responses and the peak levels of viremia postchallenge, suggesting that CTL were associated with early viral clearance (Fig. 5). In fact, the rise in measurable circulating tetramer-positive CD8<sup>+</sup> T cells appeared to occur in closer temporal association with the peak of primary viremia in the experimentally than in the control vaccinated monkeys. Importantly, no temporal association was noted between clearance of early viral replication and the emergence of CTL specific for the subdominant p68A Pol epitope, with plasma viral RNA no longer detectable by day 56 and p68A tetramer-binding cells first detected on day 42 after challenge.

Finally, the durability of the memory CTL responses elicited by recombinant canarypox was assessed. Another cohort of six *Mamu-A*01 rhesus monkeys was vaccinated with recombinant canarypox constructs vcp172-ALVAC/SIV gag-pol and vcp1420-ALVAC/IIIB gag-pol-env gp160 or vcp250-ALVAC/IIIB gag-pol-env gp120-TM at 0, 1, 6, and 12 mo. Twenty-one months following the fourth immunization, PBL were obtained from these six monkeys and stimulated in vitro with p11C peptide. Significant numbers of tetramer-binding CD8<sup>+</sup> T lymphocytes were detected in the peptide-stimulated lymphocyte populations of three of these monkeys (391L, 392L, 394L). Functional lytic activity was also evident in these lymphocyte populations using p11C-pulsed autologous B lymphoblastoid cell lines (B-LCL) as target cells (Fig. 6). Peptide-stimulated CD8<sup>+</sup> T lymphocytes of the other three monkeys also bound tetramer, albeit at a lower level, and mediated low level lysis of p11C-pulsed autologous B-LCL. Thus, the vaccine-elicited memory CTL responses in the recombinant canarypox-vaccinated monkeys were quite durable.

**Discussion**

Recent studies have shown that a variety of vaccine approaches can elicit virus-specific CTL in nonhuman primates. Experiments in rhesus monkeys have shown that such diverse approaches as immunization with the live recombinant vaccinia (34), live recombinant modified vaccinia Ankara (35), live recombinant adenovirus (P. R. Johnson, K. R. Clark, M. J. Connell, S. Robinson, D. Rohne, and N. L. Letvin, unpublished data), live recombinant poxvirus vector NYVAC (36), live recombinant gene-deleted adenovirus (T. M. Fu and J. W. Shiver, unpublished observations), and plasmid DNA (10, 37) can elicit SIV Gag p11C-specific CTL. Interestingly, comparable CTL populations were detected in the peripheral blood of recombinant canarypox-vaccinated monkeys.
and recombinant modified vaccinia Ankara-vaccinated monkeys after those lymphocyte populations were stimulated in vitro with specific Ag (32). Although high levels of circulating tetramer-positive CD8\(^+\) T lymphocytes could not be demonstrated in many of the recombinant canarypox-vaccinated monkeys before challenge, Gag and Pol epitope-specific CTL were readily seen following challenge (Fig. 3).

The quantitative assessment of CTL populations has, for the most part, relied on studies of dominant epitope-specific responses in inbred mouse strains (38, 39). Results from such studies have been generalized to make assumptions regarding the biology of all CTL responses. In beginning to explore CTL responses specific for both dominant and subdominant viral CTL epitopes in outbred nonhuman primate populations (26), it is becoming clear that the biology of these types of responses may differ. We have recently shown that the efficiency of the induction of immune responses to dominant and subdominant epitopes may be quite different for various vaccine modalities (40). In the present study, we have demonstrated that the kinetics for the expansion of memory CTL responses to dominant and subdominant epitopes may, in fact, be different. It is possible, however, that the variations in the kinetics of the emergence of these immune responses following virus infection may simply reflect differences in the magnitudes of the memory CTL populations elicited by vaccination.

CD8\(^+\) T lymphocytes have been shown to be critical for containing HIV and SIV replication, and vaccine-elicited CTL have been shown to contribute to the containment of virus replication in monkeys following SIV and SHIV infections (9, 32). Those vaccine studies in monkeys demonstrated a correlation between virus control and prechallenge tetramer-positive, circulating CD8\(^+\) T lymphocytes. The present study builds upon these observations in two potentially important ways. First, this study shows that the emergence of CTL specific for the dominant p11C epitope, but not the subdominant p68A epitope, correlates temporally with the clearance of early viral replication following challenge. The late arising CTL response to the subdominant Pol epitope in the vaccinated monkeys following viral challenge may contribute to the long-term containment of viral replication in those monkeys. This suggests that the contributions of dominant epitope-specific CTL responses may play a different role in controlling virus spread than subdominant epitope-specific CTL responses. Second, this study shows that CTL responses specific for the dominant p11C epitope rapidly expand in the experimentally vaccinated monkeys following viral challenge, even in monkeys in which vaccine-elicited CTL were not detectable before infection. This suggests that even a low frequency population of vaccine-elicited virus-specific memory CTL may expand following infection.

The Mamu-A*01\(^+\) monkeys that received the control recombinant pox vaccinations in this study all contained SIVmac replication remarkably well after rectal inoculation of the challenge virus, with no detectable virus in their plasma by day 56 postchallenge. This degree of viral containment in the control vaccinated monkeys was too great to allow a meaningful assessment of the antiviral effect of the recombinant canarypox vaccine constructs in the experimentally vaccinated monkeys. In fact, the monkeys evaluated in the present study were a subset of animals from an experiment evaluating recombinant canarypox immunization in a large group of monkeys that included Mamu-A*01\(^+\) and Mamu-A*01\(^-\) animals (33). In that study, the Mamu-A*01\(^+\) control vaccinated monkeys contained SIVmac replication better than the control vaccinated Mamu-A*01\(^+\) monkeys, suggesting that the Mamu-A*01 allelic may have conferred some degree of protection against viral replication when this particular isolate of SIVmac was inoculated by the intrarectal route.

Nevertheless, the present study clearly documents an important aspect of the immunogenicity of these vaccine constructs. It demonstrates that recombinant canarypox vaccination does prime for SIVmac Gag- and Pol-specific CTL responses that can be detected in PBL with highly sensitive assays after in vitro stimulation and after in vivo exposure to SIVmac. Whether a secondary CTL response of this magnitude can confer meaningful protection against a physiologic mucosal exposure to HIV-1 in humans of lower infectious doses of virus than used in this study can only be determined in human vaccine trials.

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