Effective Induction of Simian Immunodeficiency Virus-Specific Cytotoxic T Lymphocytes in Macaques by Using a Multiepitope Gene and DNA Prime-Modified Vaccinia Virus Ankara Boost Vaccination Regimen

TOMAS HANKE,† RACHEL V. SAMUEL,† TOM J. BLANCHARD,‡ VERONICA C. NEUMANN,‡ TODD M. ALLEN,§ JON E. BOYSON,§ SALLY A. SHARPE,§ NICOLA COOK,§ GEOFFREY L. SMITH,‡ DAVID I. WATKINS,‡ MARTIN P. CRANAGE,§ AND ANDREW J. MCMICHAEL§

Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS,† Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE,‡ and Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG,§ United Kingdom; PowderJect Vaccines, Inc., Madison, Wisconsin 53711§; and Wisconsin Regional Primate Research Center, University of Wisconsin—Madison, Madison, Wisconsin 53715§

Received 8 April 1999/Accepted 16 June 1999

DNA and modified vaccinia virus Ankara (MVA) are vaccine vehicles suitable and safe for use in humans. Here, by using a multicytotoxic T-lymphocyte (CTL) epitope gene and a DNA prime-MVA boost vaccination regimen, high levels of CTLs specific for a single simian immunodeficiency virus (SIV) gag-derived epitope were elicited in rhesus macaques. These vaccine-induced CTLs were capable of killing SIV-infected cells in vitro. Fluorescence-activated cell sorter analysis using soluble tetrameric major histocompatibility complex-peptide complexes showed that the vaccinated animals had 1 to 5% circulating CD8+ lymphocytes specific for the vaccine epitope, frequencies comparable to those in SIV-infected monkeys. Upon intrarectal challenge with pathogenic SIVmac251, no evidence for protection was observed in at least two of the three vaccinated animals. This study does not attempt to define correlates of protective immunity nor design a protective vaccine against immunodeficiency viruses, but it demonstrates clearly that the DNA prime-MVA boost regimen is an effective protocol for induction of CTLs in macaques. It also shows that powerful tools for studying the role of CTLs in the control of SIV and human immunodeficiency virus infections are now available: epitope-based vaccines, a protocol for an effective induction of CTLs in primates, and a simple and sensitive method for quantitation of epitope-specific T cells. The advantages of the DNA prime-MVA boost regimen as well as the correlations of tetramer staining of peripheral blood lymphocytes with CTL killing in vitro and postchallenge control of viremia are discussed.

Because a safe and effective vaccine remains the best hope for controlling the human immunodeficiency virus (HIV) pandemic, the development and testing of vaccines are some of the primary objectives of AIDS research. The work presented here is a part of a long-term effort to develop an HIV vaccine with a particular emphasis on the induction of cytotoxic T lymphocytes (CTLs). This is because there is growing evidence that these cells play in the fight against HIV infection. Furthermore, a reliable and effective method for CTL induction in primates would be of a great value to vaccine development in general.

Central to the evaluation of new vaccine strategies is the use of animal models. Vaccination of experimental animals permits important aspects of vaccine design and vaccination regimens to be addressed in a faster and cheaper way than human trials, and it ultimately provides a basis for a more effective planning of clinical trials. For HIV infection and AIDS, infection of nonhuman primates with immunodeficiency viruses offers a spectrum of models in terms of disease severity and difficulty in preventing virus infection. These models range from the infection of chimpanzees with HIV-1 SF2, a model in which neutralizing antibodies provide protection (6, 19, 26), to infection of rhesus macaques with simian immunodeficiency virus SIVmac, in which a role for cell-mediated immune responses in partial or complete protection is strongly supported (18, 23, 33, 42, 54). In turn, only results from clinical trials will show which of these monkey models is the closest to HIV infection of humans.

Recent advances in biotechnology have provided new tools for quantitation of the epitope-specific T-cell responses. Not long ago, detection of specific CTLs relied on standard 51Cr-release and limiting dilution assays (LDAs), which required antigen-driven expansion of T cells in vitro. These methods have tended to underestimate the numbers of CTLs. In contrast, the use of fluorochrome-labelled tetrameric major histocompatibility complex (MHC) class I-peptide complexes in fluorescence-activated cell sorter (FACS) analysis with a FACScan flow cytometer allows direct determination of MHC-peptide-specific T cells in fresh unstimulated blood (3). This technique and the gamma interferon-based enzyme-linked immunospot (ELISPOT) assay (44) have revealed impressive T-
cell responses to viral infections reflected in frequencies of virus-specific CD8+ T cells 50 to 500 times higher than that estimated originally by LDA. Thus, around 2% of circulating CD8+ T cells were HIV specific in many individuals infected with HIV (47), and up to 10% of CD8+ T cells recognized an SIV-derived epitope during persistent SIV infection of macaques (40). Here, the tetramer technology is used for the quantitation of CTL responses induced by vaccination of macaques.

The immunogen tested in this study was constructed as a string of 24 epitopes derived from HIV and SIV proteins recognized by murine, macaque, and human CTLs (32). Consequently, the same vaccines can be tested for the induction of the CTL responses in mice, monkeys, and humans. This immunogen was expressed from plasmid pTH DNA and modified virus Ankara (MVA; an attenuated vaccinia virus), i.e., vaccine vehicles that are safe and acceptable for use in humans. While both vaccines were shown to be immunogenic in mice in their own right (30–32), the most potent protocol for induction of CTLs was found to be the DNA prime-MVA boost regimen (29). This was the only protocol that fully protected mice in a malaria vaccine trial (61). Some past vaccination strategies were highly immunogenic in mice, but failed to induce comparable CTL responses in humans (72); therefore, it was important to assess the immunogenicity of the DNA prime-MVA boost regimen in nonhuman primates prior to clinical trials. Here, data are presented which demonstrate that this vaccination regimen induced high levels of SIV-specific CD8+ lymphocytes in the circulation of all immunized rhesus macaques. However, strong responses against this epitope did not protect at least two of three vaccinees against a rigorous mucosal challenge.

MATERIALS AND METHODS

Cells. Chicken embryo fibroblasts (CEF s) were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, and penicillin-streptomycin. B-lymphoblastoid cell lines (B-LCLs) were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and penicillin-streptomycin. All cells were cultured in a humidified incubator at 5% CO2 at 37°C.

Tissue typing of macaques by sequence-specific primer (SSP)-PCR and direct sequencing. Genomic DNA was prepared from whole peripheral blood by using a Puregene DNA isolation kit (Gentra Systems) according to the manufacturer’s protocol, and the Mamu-A*01 alleles were identified as described previously (38). Briefly, the Mamma-A*01-alleles were amplified by PCR from 50 to 150 ng of genomic DNA by using allele-specific primers, and positive PCR products were identified on a 1% agarose gel. Positive samples were incubated with shrimp alkaline phosphatase (AP) and exonuclease I to remove unincorporated deoxynucleoside triphosphates (dNTPs) and residual single-stranded PCR primers, respectively (U.S. Biochemicals, Cleveland, Ohio) and then were heat inactivated. The enzyme-treated, heat-inactivated PCR products were sequenced with sequencing primers and the Taq FS DyeDexony Terminator Cycle Sequencing kit (Perkin-Elmer), and the samples were analyzed with an ABI 373 automated DNA sequencer.

Preparation of the pTH DNA vaccine and immunization. The H immunogen expressed from the multiepitope DNA and MVA vaccines (32) contained an SIV gag-derived epitope, TPYDINQML, recognized by CTLs in rhesus macaques (Macaca mulatta) in the context of the Mamma-A*01 MHC class I molecule (13, 43). However, detailed characterization of the Mamma-A*01-bound peptides revealed that the optimal binding peptide was actually CTPYDINQM (p11C, C-M) (1). Therefore, both the DNA and MVA vaccines used in this study were engineered to contain the MCMV gag 20% FBS, 2 mM L-glutamine, and penicillin-streptomycin. B-lymphoblastoid medium (Gibco) supplemented with 10% fetal bovine serum (%). Cells. Cells. Cells.

Preparation of soluble tetrameric MHC-peptide complexes. DNA coding for the signal peptide-negative extracellular domains of the Mamma-A*01 MHC class I heavy chain was amplified by PCR from cDNA with the primer MamuNdeI (5′-CCG CGA CAT ATG GGC TCT CAT ATG ATG) and the 3′ primer 5′-G TGA TAA GCT TAA CGA TGA TTC CA CAC CAT TTT CTG TGC ATC AAC ATG ATG CAG GAA TTC CCA TCA CTC CAG GTC GAG G C. The former primer contained a site for restriction endonuclease NdeI and HindIII and inserted into the same sites in the pOligynex vector pGMT7. The fungus monkey coding for a signal peptide-negative β2-microglobulin was ampliﬁed by PCR from a cDNA clone by using primers DLB BACK (5′-TCA GAC CAT ATG TCT CGC TTC GCC GCC) and DLB MFOR (5′-TCA GAC AAG CCT TTA CAT GTC TGC ATG CCA C) and cloned into the NotI and HindIII sites of pGMT7. Both chains were expressed in Escherichia coli BL21, puriﬁed from inclusion bodies, denatured in 8 M urea, and refolded in the presence of His-tagged chaperones. The refolded receptor was puriﬁed with the BiA enzyme (Avi-dity) and puriﬁed on fast-performance liquid chromatography and MonoQ ion-exchange columns. The amount of biotinylated refolded MHC-peptide complexes was estimated in an enzyme-linked immunosorbent assay, whereby monomeric complexes were captured by formation-sensitive monoclonal antibody (MAb) W6/32 and detected by AP-conjugated streptavidin (ExtrAvidin; Sigma) to the refolded biotinylated MHC-peptide complexes was estimated in an enzyme-linked immunosorbent assay, whereby monomeric complexes were ﬁrst captured by formation-sensitive monoclonal antibody (MAb) W6/32 and detected by AP-conjugated streptavidin (Sigma) followed by a colorimetric reagent for AP. The formation of tetrameric complexes was induced by addition of phycocerythrin (PE)-conjugated streptavidin (ExtrAvidin; Sigma) to the refolded biotinylated monomers at a molar ratio of MHC-peptide monomer–PE-streptavidin of 4:1. Streptavidin was added in 1/10 of the ﬁnal amount separated by a 20-min incubation at 4°C. PE-tetrameric complexes were stored in the dark at 4°C until use.

FACS analysis. Aliquots of Lymphoprep cushion-isolated PBLs were stored at –70°C until use. On the day of analysis, PBLs were thawed, washed once with PBA (PBS, 1% bovine serum albumin, 0.1% sodium azide), incubated with 1 μg of PE-conjugated tetrameric Mamma-A*01-Gag peptide complexes, first at 4°C for 20 min and then on ice for 5 min. One microgram of Tri-color-conjugated antihuman CD8 MAb (Caltag) was added, and this mixture was then incubated with the cells on ice for 20 min. Cells were washed twice with PBA before being frozen in formaldehyde (PBS, 1% paraformaldehyde, 2.5% PBS). Two-color cytofluorometric analysis was performed with a Becton Dickinson FACS Vantage. Forward and compensation levels were determined by using both unstained lymphocytes for setting the background levels of fluorescence to the first log of the scale and single PE- and Tri-color-stained lymphocytes for adjusting the spectral overlap. All time points from each animal were analyzed with the same FACS settings. A gate was applied to the live lymphocytes. Data analysis and presentation were done with the CellQuest software (Becton Dickinson).

SIV challenge. Animals were challenged intrarectally (i.r.) with 20 i.r. median lethal doses of cell supernatant of pathogenic SIVmac251 grown in simian PBLs kindly provided by A.-M. Aubertin, Université Louis Pasteur, Strasbourg, France.
Plasma RNA load. The number of SIV genome equivalents per 1 ml of peripheral blood was determined by quantitative competitive reverse transcription (RT)-PCR method as described before (66). A calibrated amount of internal standard RNA was added to and copurified with the sample RNA to be analyzed before RNA purification to compensate for sample degradation and amplification efficiency. The limit of detection was 40 genome equivalents per ml of plasma.

Cell-associated virus load. Freshly Lymphoprep-separated lymphocytes were cocultivated in duplicates with the human T-cell line C8166 at either a single dose of 10^5 cells or additionally fivefold diluted from 4 \times 10^5 to 130 cells in duplicates. Medium and C8166 cells were replenished every 3 to 4 days, and the total culture volume was maintained at approximately 15 ml. All cultures were observed for 28 days or until a cytopathic effect was apparent. The assay limit was set to be 1 infected cell per 10^6 PBLs. Culture results were confirmed by indirect immunofluorescent staining. Fifty percent end points were calculated by using the Kärber formula, and the results were expressed as the number of infected cells per 10^6 PBLs. The lack of SIV infection was confirmed by a negative nested PCR for proviral DNA.

CD4^+ and CD8^+ lymphocyte enumeration. A whole-blood lysis method was used to assay PBLs for CD4^+ (OKT4; Ortho Diagnostics) and CD8^+ (DK25; Dako) cells (17). Briefly, heparinized blood samples were combined with a specific or isotype-matched MAbs, incubated with Immunolyse (Coulter) to lyse erythrocytes, fixed with 1% formaldehyde in PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson). Absolute numbers of each lymphocyte subset were determined from the differential leukocyte counts and total leukocyte counts.

Animal care. Indian rhesus macaques were bred and immunized at the Oxford University Farm and challenged at the Centre for Applied Microbiology and Research in Porton Down in accordance with the guidelines of the Home Office Code of Practice. All procedures were carried out under general anesthesia.

RESULTS

DNA prime-MVA boost regimen induced strong CTL responses capable of killing SIV-infected PBLs. Mamu-A*01-positive rhesus macaques Cyd, Di, and Doris were vaccinated by the DNA prime-MVA boost regimen (29) modified for primates. The animals were immunized twice i.d. with a total of 8 \mu g of pTH.HW DNA per immunization with the Dermal XR (formerly Accell) gene delivery device of PowderJect Vaccines, Inc. (weeks 0 and 8), followed by two i.d. needle injections of 5 \times 10^8 PFU of MVA.HW (weeks 17 and 22) administered into the proximal area of the right hip. Both the DNA and MVA vaccines were safe and did not cause any obvious adverse reactions or discomfort.

Blood from each animal was drawn at multiple time points throughout the immunization schedule. PBLs were isolated on a Lymphoprep cushion, restimulated with peptide in vitro, and tested for their cytolytic activity against peptide-pulsed autologous B-LCL cells as targets. The assay was carried out on day 14. (A) The restimulated effectors were incubated for 6 h with 51Cr-labelled peptide or unpulsed (the first two bars for each animal) or pulsed (the last two bars for each animal) autologous B-LCL cells at the effector/target ratios indicated below the graphs. (B) Restimulated PBLs drawn from Doris on week 35 were tested on 51Cr-labelled autologous B-LCL cells (left) or 6-day PHA-stimulated PBLs (right), which had been treated as indicated, at effector/target ratios of 54:1, 27:1, and 13:1.

Figure 1. Cytolytic T-cell responses determined by a standard 51Cr-release assay. PBLs were isolated, and the bulk CTL cultures were treated in vitro with the following reagents: peptide CTPYDINQM and IL-7 on day 0, Lymphocult-T on day 3, peptide-pulsed irradiated autologous B-LCL on day 8, and Lymphocult-T on day 11. The CTL assays were carried out on day 14. (A) The restimulated effectors were incubated for 6 h with 51Cr-labelled peptide or unpulsed (the first two bars for each animal) or pulsed (the last two bars for each animal) autologous B-LCL cells at the effector/target ratios indicated below the graphs. (B) Restimulated PBLs drawn from Doris on week 35 were tested on 51Cr-labelled autologous B-LCL cells (left) or 6-day PHA-stimulated PBLs (right), which had been treated as indicated, at effector/target ratios of 54:1, 27:1, and 13:1.
In these animals, DNA immunization alone did not elicit any population with above-background tetramer staining; however, the first MVA immunization stimulated frequencies ranging from 0.4 to 3% of CD8$^+$ cells (Fig. 3A). In Di and Doris, these levels were boosted further by the second MVA vaccine up to 4.9 and 1.5%, respectively. The frequencies peaked 1 week after the MVA vaccination and decreased to one-half within approximately 1 week. A significant tetramer staining was still observed in Di and Doris 17 weeks after the last MVA immunization (week 35).

Accelerated schedule of immunization induced high levels of CD8$^+$ p11C, C-M-specific PBLs. The 5- to 8-week intervals between individual immunizations used in the first group of macaques were based on experiments which employed the gene gun to elicit antibody responses (20). When a faster, 3-week-interval vaccination schedule was tested, similar levels of tetramer-specific CD8$^+$ cells were induced, although with a seemingly slower kinetics (Fig. 3B). The peak vaccine-induced lymphocyte frequencies were detected in 2 or 3 weeks rather than 1 week after the MVA immunization. One animal, Fiona, had a positive tetramer staining 2 weeks after the second DNA vaccine (Fig. 3B).

DNA vaccine primes CTL responses. To assess whether or not the DNA vaccine primes CTL immune responses and therefore is an important part of the vaccination regimen, two animals, Emer and Fidel, were immunized with the MVA vaccine alone, and the induction of the Mamu-A*01–p11C, C-M-specific T cells in their circulation was determined by tetramer staining (Fig. 3C). The most striking observation suggesting that DNA had made a difference was that in these two animals, two MVA vaccinations were required to detect significant tetramer staining. This is similar to previously published results (63). Thus, these data, the DNA-induced tetramer staining in Fiona (Fig. 3B), and CTL lysis after a 3-week peptide restimulation in vitro (2) indicate that there was priming of CTLs by a gene gun-delivered DNA vaccine.

One out of three animals remained uninfected after a pathogenic i.r. challenge. Animals Cyd, Di, and Doris were boosted with 5 × 10$^8$ PFU of MVA.HW i.d. at the times indicated below each graph. Frozen isolated PBL samples were thawed, double stained with PE-conjugated tetrameric MHC-peptide complexes, and human CD8-specific Tri-color-conjugated MAb for the CD8 marker, and examined by FACS. An n indicates a sample measured, but negative.

The third dose of recombinant MVA applied to the same site expanded the CD8$^+$ Mamu-A*01–p11C, C-M-specific
population to the highest levels seen during the first two MVA vaccinations (Fig. 3A and 5). Thus, any anti-MVA responses (not actually measured) did not prevent an efficient boost with the recombinant MVA-expressed immunogen. At the same time, the tetramer staining did not increase above the previous levels, so it could also be argued that following a DNA priming, the “ceiling” of MVA-elicited responses was reached with one (Cyd) or two (Di and Doris) MVA doses.

The SIV challenge infected Di and Doris, while Cyd remained SIV negative by both plasma RNA and cell-associated viral loads (Fig. 4A and B, left panels). All four naive controls became infected (Fig. 4A and B, right panels). During the first weeks following SIV challenge, absolute CD4⁺ and CD8⁺ cell counts increased transiently in all monkeys without any noticeable correlation with protection or infection (Fig. 4C and D). The postchallenge tetramer staining of uninfected Cyd’s PBLs did not show any expansion of Mamu-A*01–p11C, C-M-specific cells (Fig. 5). In contrast, the SIV challenge drove the increment in tetramer staining of PBLs of the infected Di and Doris. The temporal relationship between the Mamu-A*01–p11C, C-M-specific CD8⁺ cells and virus loads was consistent with the contribution of these cells to the control of the acute infection (60), after which the levels of tetramer staining decreased, but remained relatively high, presumably due to the continuous presence of replicating virus. No p11C, C-M-specific CTL escape mutants were observed in the vaccinated and infected animals in a total of 14 individually cloned PCR products derived from two independent amplifications of PBL DNA at week 16 postchallenge. Interestingly, the uninfected macaque, Cyd, showed the highest cytotoxic activity in vitro of peptide-restimulated PBLs drawn 4 weeks after the challenge (Fig. 6), which may imply the strongest long-term CTL memory among the three challenged animals.

**DISCUSSION**

It was demonstrated previously in mice that a particular sequence of vaccinations, DNA followed by MVA, elicited high levels of CTLs (29) and conferred complete protection against *Plasmodium berghei* sporozoite challenge (61). Here, immunization of macaques by using a similar DNA prime-MVA boost vaccination regimen generated strong CTL re-
effector/target ratios were 25:1, 12:1, and 6:1.

What makes the DNA prime-MVA boost regimen efficient in induction of CTLs? The exact mechanisms are not known, but several factors may be relevant. First, priming of CTL responses by using DNA as a vaccine vehicle may allow the initial responses to be focused on the recombinant immunogen, simply because that is the only foreign protein expressed. Although recombinant poxviruses may be intrinsically more immunogenic than DNA vectors as vaccine vehicles, virus-infected cells must produce a large number of peptide epitopes derived from virus proteins and presented by MHC class I. These may compete with the recombinant immunogen for CTL immunodominance. In most previous studies, only a minority of vaccinees immunized with recombinant vaccinia virus responded to the inserted protein, although all responded to the vaccinia virus antigens (23, 27). In this regimen, DNA-primed animals have already increased frequencies of immunomod-specific CTL precursors or memory when vaccinated with MVA. Second, both the DNA and MVA vaccines were delivered into the dermis, a major immunological inductive site, in which the multiepitope immunogen is produced by keratinocytes and shed into the immediate vicinity of Langerhans cells (LCs) and/or the multiepitope gene is delivered directly into the LCs (50). Upon activation, LCs migrate to the draining lymph nodes, where they serve as the most efficient initiators and modulators of immune reactions (4). In mice, the i.d. route of immunization was more efficient than the intramuscular route of infection (61).

The use of MVA rather than other vaccinia virus strains or poxviruses may be relevant (61), possibly because MVA has lost several genes coding for immunomodulatory proteins, such as the soluble receptors for gamma interferon, alpha/beta interferon, tumor necrosis factor, and CC-chemokines (8). MVA also generates a strong type I interferon response, which is likely to contribute to the CTL induction (67). Fourth, both DNA and MVA mimic natural infection by expressing antigens endogenously and thus deliver them to the MHC class I processing and presentation pathway. Fifth, the expressed HIV multi-CTL epitope is nontoxic to the transfected cells, which may be due to the sequestration of the polypeptide into large cytoplasmic vesicles (32). This may allow the cells to sustain the production of the immunogen over a longer time or until their destruction by the immune responses. Finally, there is now an emerging body of data suggesting that combined vaccination protocols involving different vaccine vehicles, routes, or means of antigen presentation to the immune system induce immune responses more efficiently and in some instances protected macaques against virus challenge. Thus, DNA priming followed by Env protein boosting increased antibody, but not CTL, responses and protected macaques against a nonpathogenic simian-human immunodeficiency virus strain (SHIVHXBc2) challenge (41). Sequential bimodal immunizations of monkeys with DNA and the recombinant Western Reserve (WR) strain of vaccinia virus significantly decreased virus loads after infection (21). DNA-prime-recombinant fowlpox boost vaccinations held nonpathogenic HIV (36) and nonpathogenic SHIVHXBc2 (54) below the level of detection. In the latter study, two animals remained uninfected after a challenge with pathogenic SHIVsoa,GPR a protection mediated by a neutralizing antibody-independent mechanism; however, there is a possibility that protective responses could have been boosted or induced by the two previous SHIVHXBc2 exposures.

Mamu-A*01–p11C, C-M-specific CD8\(^+\) PBLs are functionally heterogeneous and consist mainly of two subpopulations. The first subpopulation are memory or precursor CTLs which may be fewer in number, but are able to proliferate and need to be reactivated and expanded to observe killing in vitro. Their frequencies can be determined in LDAs. The second subpopulation are the expanded CTLs, which if sufficiently high can be demonstrated by fresh killing (2). However, antigen-specific CD8\(^+\) T cells can also be “silenced” or rendered nonfunctional, but are still abundant enough to be detected by a tetramer (77). On a few occasions the present study demonstrated discordance between CTL lysis in vitro and tetramer staining. First, Cyd, Di, and Doris gave respective peptide-specific lyases of 48, 50, and 66% of total \(^{51}\)Cr release at week 23 (Fig. 1), while their tetramer-detected CD8\(^+\) T-lymphocyte levels were 0.7, 4.9, and 1.5% of total CD8\(^+\) PBLs, respectively (Fig. 3). Similarly, 49, 38, and 34% specific lysis at week 49 (Fig. 6) corresponded to respective 0.52, 4.90, and 2.46% tetramer-stained CD8\(^+\) PBLs (Fig. 5). A closer comparison of the immune responses in Doris at weeks 23 and 35 (Fig. 1) revealed that while the peptide-specific lysis of the same effector/target ratios decreased 1.5-fold (28:1) and 3-fold (14:1), the tetramer staining decreased 7-fold (Fig. 3A). Also, while in all animals the tetramer staining decreased at least by half between weeks 18 and 19 (Fig. 3), the peptide-specific lysis after in vitro restimulation increased (not shown). In the absence of persisting high levels of antigen due to the lack of MVA replication, the half-life of the tetramer staining was approximately 1 to 2 weeks (Fig. 3A and S [Cyd]). Finally, no correlation between tetramer staining and virus load could be inferred, although the numbers of animals in this study were small.

Can CTLs protect against infection with immunodeficiency viruses? Cytolytic activity cannot prevent incoming cell-free virus from infecting host cells. However, in the laboratory, CTLs killed HIV-infected cells before they produced new virions (74) and released chemokines which inhibited HIV infection (51, 68). Thus, in vivo, CTLs may be able to clear the initial small number of infected cells before HIV spreads further and establishes generalized infection (46, 73). This might explain detection of HIV-specific CTL responses in exposed, but uninfected commercial sex workers whose cells were fully susceptible to infection with HIV (56, 57), in uninfected infants born to HIV-infected mothers (59), and seronegative health care workers occupationally exposed to HIV-contaminated body fluids (49). The same SIV gag-derived p11C, C-M epitope as used in this study was shown to be immunodominant (40)
and to contribute to the suppression of both the acute and chronic viremias (60) in SIV-infected monkeys. There have been two studies in which animals with vaccine-induced CTLs against the same single Gag epitope were challenged and not protected against infection (75), although a decrease in virus load was observed (45). However, there was a possibility that the levels of CTLs in the former challenge study were lower than those in the animals here due to boosting with a suboptimal 12-mer peptide, p11C (64), and protection is likely to depend on the strength of CTLs (23). Alternatively, CTLs specific for some SIV epitopes other than p11C, C-M may be more protective by analogy to the infection with lymphocytic choriomeningitis virus, in which CTLs specific for different epitopes differed in their protective capacities (24, 25). Thus, CTLs against proteins expressed early in the replication cycle (e.g., Nef, Tat, or Rev), may have a better chance to prevent infection than those against late structural proteins (e.g., Gag), although this difference is likely to be subtle.

More importantly, broader CTL responses against multiple epitopes may be critical for the success of a prophylactic HIV vaccine. Three rhesus epitopes restricted by Mamu-A*01, -A*02, and -B*01 molecules had been published (43, 70, 76) at the time of the construction of the DNA and were included in the HW multi-CTL epitope vaccines employed in this study. However, only the Mamu-A*01-restricted epitope turned out, after a correction (1), to be immunogenic in this study. Some uncertainty surrounds the Mamu-B*01-restricted epitope, because it does not fit the amino acid sequence motif currently defined for this MHC type (71). None of the three selected Mamu-A*01+ animals was Mamu-A*02+. Recently, six new epitopes and their restricting Mamu molecules have been defined, and sequence-specific primers have been designed to screen for the Mamu-A*01 (38) and several other alleles (71). These new developments increase the utility of the rhesus macaque model for testing epitope-based vaccines.

CTLs require “help” provided by CD4+ T cells for maintenance of their effector functions, and even dendritic cells may be conditioned by CD4+ cells to provide sufficient costimulatory signals to CTLs (5, 52, 62). For CTL epitope-based vaccines, such as the HIV polypeptide used in this study, a long string of MHC class I-restricted epitopes will create novel helper epitopes presented by MHC class I molecules and activate CD4+ T-cell responses after vaccination. However, these CD4+ helper T cells will not be activated by incoming virus. Should a prophylactic vaccine induce HIV-specific help? Delay in CD4+ T-cell activation may slow the initial spread of HIV, because HIV replicates in activated CD4+ cells. It has been shown in HIV-infected persons that vaccination with a common recall antigen, tetanus toxoid, increased plasma virus load (48, 65). Vigorous Gag-specific CD4+ T-cell responses have been associated with the control of viremia in chronic HIV infection and may enhance CTL responses (55). Some (14, 15, 35), but not all (55), studies have found CD4+ T-cell responses in exposed seronegative individuals, which might contribute to their lack of infection (22). Comparison of protective effects by vaccination with poly-CTL epitopes and whole proteins, which contain virus-derived MHC class II epitopes, could determine how important the CD4+ T-cell responses are in prophylactic immunity (34).

In this challenge, one out of three DNA-MVA-vaccinated animals challenged 1 week after the last MVA immunization remained uninfected, while all four naive animals succumbed to infection (Fig. 4). As a part of a parallel, but separate experiment, four control macaques were immunized three times with 10⁸ PFU of wild-type (no SIV-derived gene) MVA at 12-week intervals and challenged 2 months after the last MVA immunization. One of these animals remained uninfected (data not shown [16]). In another study, wild-type MVA immunization suppressed virus loads in animals challenged with SIV 1 month after the last MVA vaccine (7). Taken together, these results suggest that MVA may induce a non-specific antiviral effect, the mechanism of which is currently under investigation. Although for use in humans, determination of the underlying mechanisms would be comforting, the antiviral effects of MVA are generally welcome.

In conclusion, only one out of three immunized monkeys remained uninfected after a challenge which was at the pathogenic and hard-to-protect end of the monkey model spectrum. Based on the information available, this protection could not be clearly correlated with the p11C, C-M-specific T lymphocytes in the blood at the time of the i.r. challenge. It should also be noted that the challenge dose, even though it may not have infected all animals, is likely to be much higher than the doses in natural HIV transmission. In any case, we and others believe that the high levels of vaccine-induced CTL responses in macaques merit an evaluation of the immunogenicity and, if that is satisfactory, efficacy of similar vaccines in clinical trials.

ACKNOWLEDGMENTS

We thank Eric Prieur for the preparation of CEF cells; Susan Steffen for tissue typing of rhesus macaques; Rod Dunbar and Graham Ogg for advice during the production of tetramers; Sharon Leech for assistance with virus load detection; and Sarah Wolfensohn, Maggie Lloyd, James Davys, and Mike Dennis for excellent veterinary services. Grant support from MRC U.K. and the International AIDS Vaccine Initiative (T.H., R.V.S., and A.J.M.); MRC U.K. (T.J.B. and G.L.S.), grants R00167, AI32426, and AI41913 (J.E.B., T.M.A., and D.I.W.); and the U.K. Department of Health (S.A.S., N.C., and M.P.C.) is fully acknowledged.

REFERENCES

7. Biberfeld, G. Personal communication.


38. Norley, S. Personal communication.


