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Induction of simian immunodeficiency virus (SIV)-specific CTL in rhesus macaques by vaccination with modified vaccinia virus Ankara expressing SIV transgenes: influence of pre-existing anti-vector immunity

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A major aim in AIDS vaccine development is the definition of strategies to stimulate strong and durable cytotoxic T lymphocyte (CTL) responses. Here we report that simian immunodeficiency virus (SIV)-specific CTL developed in 4/4 macaques following a single intramuscular injection of modified vaccinia virus Ankara (MVA) constructs expressing both structural and regulatory/accessory genes of SIV. In two animals Nef-specific responses persisted, but other responses diminished and new responses were not revealed, following further vaccination. Vaccination of another two macaques, expressing Mamu A*01 MHC class I, with MVA constructs containing *nef* and *gag–pol* under the control of the moderate strength natural vaccinia virus early/late promoter P7.5, again induced an early Nef-specific response, whereas responses to Gag remained undetectable. Anti-vector immunity induced by this immunization was shown to prevent the efficient stimulation of CTL directed to the cognate Gag epitope, p11C C-M, following vaccination with another MVA construct expressing SIV Gag–Pol under a strong synthetic vaccinia virus-specific promoter. In contrast, vaccination of a previously unexposed animal resulted in a SIV-specific CTL response widely disseminated in lymphoid tissues including lymph nodes associated with the rectal and genital routes of SIV entry. Thus, despite the highly attenuated nature of MVA, repeated immunization may elicit sufficient anti-vector immunity to limit the effectiveness of later vaccination.

Introduction

In the face of the continued advance of the pandemic of human immunodeficiency virus (HIV), a safe effective vaccine remains the best prospect for control. The increasing body of evidence suggesting that cytotoxic T lymphocytes (CTL) play a central role in controlling viraemia (reviewed by Goulder *et al.*, 1999) has led to a wide acceptance that an effective HIV vaccine should stimulate the generation of this cell population (Letvin, 1998).

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Vaccination of macaques with live attenuated simian immunodeficiency virus (SIV) confers a high degree of protection against subsequent challenge. Notably, this experimental approach stimulates CTL responses directed against many virus-encoded proteins and elicits protection against both systemic and mucosal challenge (reviewed by Ruprecht, 1999). Thus, in designing clinically acceptable strategies for the delivery of HIV/SIV immunogens, a primary aim is to evaluate the ability of candidate vaccines to induce broad, persistent and disseminated responses that emulate those induced by live attenuated virus.

Immunization with viral vectors, particularly recombinant vaccinia viruses, has been shown to efficiently prime and

stimulate CTL responses (Bennink *et al*., 1984; Wiktor *et al*., 1984). Vectors of this type are among the most potent for inducing protective immunity in animals against viral infections and cancer (Moss, 1991; Paoletti, 1996). Indeed, the protective potential of vaccinia virus constructs expressing SIV genes has been demonstrated in the macaque model (Gallimore *et al*., 1995; Hu *et al*., 1992). The potential virulence of replication competent vaccinia virus in immunocompromised individuals, however, limits its potential for use in HIV endemic areas. In contrast, a highly attenuated vaccinia virus strain, modified vaccinia virus Ankara (MVA), has been shown to be avirulent in immunosuppressed animals and most importantly to be safe with no adverse side-effects when used in over 120000 humans at the end of the smallpox eradication campaign (Mayr *et al*., 1975). Although MVA grows well on primary chicken embryo fibroblasts (CEF), it is incapable of producing mature virions in most mammalian cells due to a block at the stage of virion formation (Meyer *et al*., 1991; Sutter & Moss, 1992). The resulting abortive infection does, however, result in DNA replication and the synthesis of both late and early proteins leading to recombinant protein expression at a level equivalent to that of a fully replication competent vaccinia virus (Sutter & Moss, 1992).

The protective potential of recombinant MVA-based vaccines against SIV has been shown in macaques where immunization with MVA expressing SIV structural genes followed by immunization with inactivated whole virus led to a reduction in virus replication after challenge with SIV (Hirsch *et al*., 1996).

As part of a European Concerted Action to determine the immunogenicity and protective efficacy of MVA constructs expressing both regulatory/accessory and structural genes of SIVmac 32H (Rud *et al*., 1994), we determined CTL responses in vaccinated macaques. Systemic SIV-specific CTL responses were measured in peripheral blood mononuclear cells (PBMC) following mitogen-driven expansion of precursors. In addition, in two Mamu A*01 MHC class I-positive macaques, CTL responses against the cognate *gag*-encoded p11C C-M epitope (Allen *et al*., 1998) were determined following peptide-driven expansion of precursors. Failure to detect such responses following initial immunization with the MVA *gag–pol* construct afforded the opportunity to compare, using a second, efficiently expressing construct, the stimulation of Gag-specific CTL responses, both systemically and locally in animals previously exposed to the MVA vector with responses generated in the absence of pre-existing anti-vector immunity.

Methods

■ **Animals.** Juvenile rhesus macaques used in this study were bred within the United Kingdom and housed according to the Home Office Code of Practice. Animals were anaesthetized by intramuscular injection with ketamine hydrochloride (Vetalar, Park Davis) for all procedures requiring removal from their cages, including femoral venepuncture and immunization.

Animals with the Mamu A*01 tissue type were selected for this study either by screening by cytotoxicity assay as previously described by Vogel *et al*. (1995), or by sequence-specific primer (SSP)-PCR and direct sequencing as previously described by Knapp *et al*. (1997).

■ **Recombinant MVA constructs and immunization.** The first set of recombinant MVA constructs was engineered to express the *tat*, *rev*, *nef*, *gag–pol* and *env* genes of SIVmac251 32H (pJ5) (Rud *et al*., 1994) under the transcriptional control of the natural vaccinia virus early/late promoter P7.5 (*tat,rev*, *nef*, *gag–pol*) or the strong synthetic vaccinia virus early/late promoter (*env*) (Chakrabarti *et al*., 1997). Briefly, the *tat*, *rev*, *nef* and *gag–pol* SIV coding sequences were placed into the unique *Sma*I site of the MVA vector plasmid pUCII-LZ-P7.5 which targets the site of deletion II within the MVA genome for insertion of recombinant genes (Sutter *et al*., 1995). The SIV *env* gene was inserted at the site of deletion III within the *Hin*dIII A-fragment of the MVA genome. Recombinant MVA were generated in CEF after transfection of the plasmid vectors, simultaneous infection with non-recombinant MVA, and screening for co-expression of the *E. coli lacZ* marker gene as described previously (Sutter & Moss, 1992). SIV transgene expression was determined by Western blot analysis for Env, Gag, Nef and Rev and the production of SIV Tat was detected by transcriptional activation. Gag–Pol expression was confirmed by detection of reverse transcriptase activity and electron microscopy for virus-like particles in tissue culture supernatants (G. Sutter & V. Erfle, unpublished data). MVA-SIVmacJ5 *gag–pol* vaccine stock was produced after large-scale virus amplification on CEF and provided through Programme EVA, Centralised Facility for AIDS Reagents, NIBSC, Herts UK.

A second MVA vector vaccine was based on a recombinant virus expressing the SIVsmH4 *gag–pol* coding sequences (nucleotides 1049–5397) regulated by the strong synthetic vaccinia virus early/late promoter (Chakrabarti *et al*., 1997) and was generated as described previously (Seth *et al*., 1998).

Monkeys were immunized by injection into different sites within the gluteal muscle on each occasion with 10^8 infectious units of each recombinant MVA in 500 µl sterile PBS. In the first experiment, four macaques were immunized at weeks 0, 12 and 24 with MVA constructs encoding SIVmacJ5 genes and a further four animals were similarly vaccinated with non-recombinant MVA (MVA-WT). In the second experiment, two macaques were immunized with MVA-SIVmacJ5 *gag–pol* and MVA-SIVmacJ5 *nef* at weeks 0, 12 and 24 followed by immunization with SIVsmH4 *gag–pol* at weeks 51 and 60.

■ **Preparation of mononuclear cell (MNC) suspensions from blood and tissues.** PBMC were isolated by Ficoll-Paque (Pharmacia) density centrifugation from heparinized blood and washed in medium consisting of RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (50 U/ml)-streptomycin (50 µg/ml) and 10% heat-inactivated foetal bovine serum. Splenic MNC and lymph node MNC were obtained by teasing these tissues and washing cells in culture medium. Splenic MNC were purified on Ficoll-Paque cushions. Jejunal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were obtained by mechanical and enzymatic dissociation using previously described methods (Polyanskaya *et al*., 2001). More than 95% of the cells obtained from the gut were viable as determined by trypan blue exclusion. Recovered cells were subsequently cultured in medium as above supplemented with 80 µg/ml gentamicin, 25 mM HEPES buffer, 0·5 µg/ml amphotericin B, 0·05 mM 2-mercaptoethanol and 5 IU/ml human recombinant interleukin 2 (IL-2, MRC AIDS Reagent Project, ADP901).

■ **Cytotoxic T cell analysis, bulk culture and chromium release assay.** To analyse Tat-, Rev-, Nef-, Gag- and Env-specific CTL activity, isolated PBMC at 2×10^6 cells/ml were incubated in culture medium

containing concanavalin A at 10 μg/ml (Sigma). On day 3, Lymphocult T (Biotest) was added to a final concentration of 10 IU/ml. Cytolytic analysis was carried out at day 7 using a standard chromium release assay. Effector cells were washed three times and tested in triplicate against chromium-labelled target cells. Autologous, herpesvirus papio-transformed B-lymphoblastoid cell lines (B-LCL) were used as targets following overnight infection with recombinant vaccinia virus expressing SIVmacJ5 Tat (vv9015), Rev (vv9005), Nef (vv9011), Gag–Pol (vv9019) and Env (vv9002) (MRC AIDS Reagent Project) or influenza virus nucleoprotein PB2 (a kind gift from F. Gotch, London, UK) at 5 p.f.u. per cell. Infected B-LCL targets were labelled with 150 μCi ^{51}Cr (Amersham) for 2 h, washed three times and 5000 cells added to each test well. Vaccinia virus-specific CTL activity was reduced with cold target competition by the addition of 50000 cells per well infected with recombinant vaccinia virus expressing SIVmacJ5 Vif (vv9003, MRC AIDS Reagent Project).

To assess Gag-specific CTL activity in Mamu A*01$^+$ animals, isolated MNC were cultured at 4×10^6 cells/ml in medium containing Gag p11C C-M peptide (CTPYDINQM) (Miller *et al.*, 1991; Allen *et al.*, 1998) at 20 μg/ml. Lymphocult T was added to a final concentration of 10 IU/ml on days 3 and 7. CTL were restimulated *in vitro* on day 7 by the addition of mitomycin C (25 μg/ml, 1 h)-inactivated, peptide-pulsed B-LCL at a ratio of 10 stimulator:1 effector cell. At day 13, effector cells were assessed for their ability to lyse Gag peptide-pulsed or untreated target cells at various effector to target ratios (E:T) in a standard chromium release assay.

Chromium release was determined in supernatants harvested after 4 h. Spontaneous release and total release from target cells were determined from wells containing target cells and medium alone or with 5% Triton X-100. The percent specific lysis was determined from the equation: % specific lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release was always < 20% of maximum release. Responses were considered positive if they were at least 10% specific lysis above that obtained from control target cells.

■ **Tetrameric MHC-peptide staining.** Mamu A*01-Gag p11C C-M peptide tetrameric complexes were prepared as described previously (Hanke *et al.*, 1999). PBMC from vaccinated macaques and from an SIV-infected macaque (positive control) were incubated with 1 μg of PE-conjugated tetramer for 20 min at 4 °C followed by 5 min on ice. Labelled anti-human CD8 antibody (Caltag) was added and incubated on ice for a further 20 min. After washing, cells were fixed in PBS containing 1% paraformaldehyde and 2·5% foetal bovine serum and stained cells were enumerated by flow cytometry as described previously (Hanke *et al.*, 1999).

■ **Assay of anti-vector antibodies.** Anti-vaccinia serum antibody responses were measured by ELISA with a vaccinia ELISA antigen (Churchill Applied Biotechnology) using standard methods. Bound antibody was detected using rabbit anti-monkey IgG-HRP conjugate (Sigma). Neutralizing antibodies were determined by plaque reduction using standard methods. Titres were calculated as the serum dilution resulting in a 50% reduction in plaque numbers.

Results

SIV-specific CTL responses following immunization with MVA-SIVmacJ5 constructs

Initially, eight rhesus macaques were immunized with MVA. Four received 1×10^8 infectious units of each MVA construct containing SIV genes, by intramuscular inoculation at weeks 0, 12 and 24. Another four animals were similarly vaccinated with MVA-WT. All animals immunized with MVA-SIV had SIV-specific CTL detectable 4 to 8 weeks after the first immunization (Fig. 1), whereas responses were not detected in the four animals immunized with MVA-WT. In general, the strongest responses were seen against Nef, to which all four MVA-SIV vaccinates responded, whilst responses to Rev and Tat were detected in two animals (V73 and V42). Further vaccination failed to reveal CTL to other SIV antigens and in animals V73 and V42 did not boost Nef reactivity. After three immunizations only animals V66 and V13 had detectable SIV-specific CTL. Not all specificities could be assayed for on each occasion due either to limited cell recovery from blood or, in the case of animal V13, to lack of sufficient autologous target cells. Nonetheless, these results showed that a single vaccination with MVA-SIV was sufficient to generate SIV-specific CTL and suggested that boosting was not efficient.

The generation of CTL was then studied in more detail in a further two macaques, 65T and 68T, with a view to examining both systemic and local responses. These animals were chosen since they had the Mamu A*01 MHC class I phenotype that restricts a well described SIV Gag CTL epitope, thus enabling efficient peptide-specific restimulation of CTL precursors and precise determination of effector activity. The two animals were vaccinated as described above with MVA-SIVmacJ5 *gag–pol* and MVA-SIVmacJ5 *nef*. The latter construct was included as this appeared to be the most immunogenic and anti-Nef CTL responses have previously been associated with protection (Gallimore *et al.*, 1995). Both animals had Nef-specific CTL activity at 2 weeks after the first immunization but this activity declined subsequently despite further immunization (Fig. 2). The specificity of the response was confirmed by the demonstration that cytolytic activity was restricted to autologous target cells.

Surprisingly, stimulation of isolated PBMC *in vitro* with the Gag p11C C-M peptide failed to reveal Gag-specific CTL on any occasion (see Fig. 4). Staining of unstimulated PBMC, taken 2 weeks after the second and 2 weeks after the third immunizations, with Mamu A*01-p11C, C-M peptide tetrameric complexes also failed to reveal SIV-specific T cells in the CD8$^+$ fraction, confirming the result obtained in the lytic assay.

Gag expression from the MVA SIVmacJ5 *gag–pol* construct

Taken together, the results obtained thus far suggested that the MVA SIVmacJ5 *gag–pol* construct was poorly immunogenic. Further investigation revealed that, whilst the original seed stock of virus efficiently produced Gag-derived virus-like particles following *in vitro* infection of cells (data not shown), large scale expansion of the vaccine stock for multi-centre vaccination experiments had resulted in reduced recombinant gene expression. Electron microscopy of cells infected at high

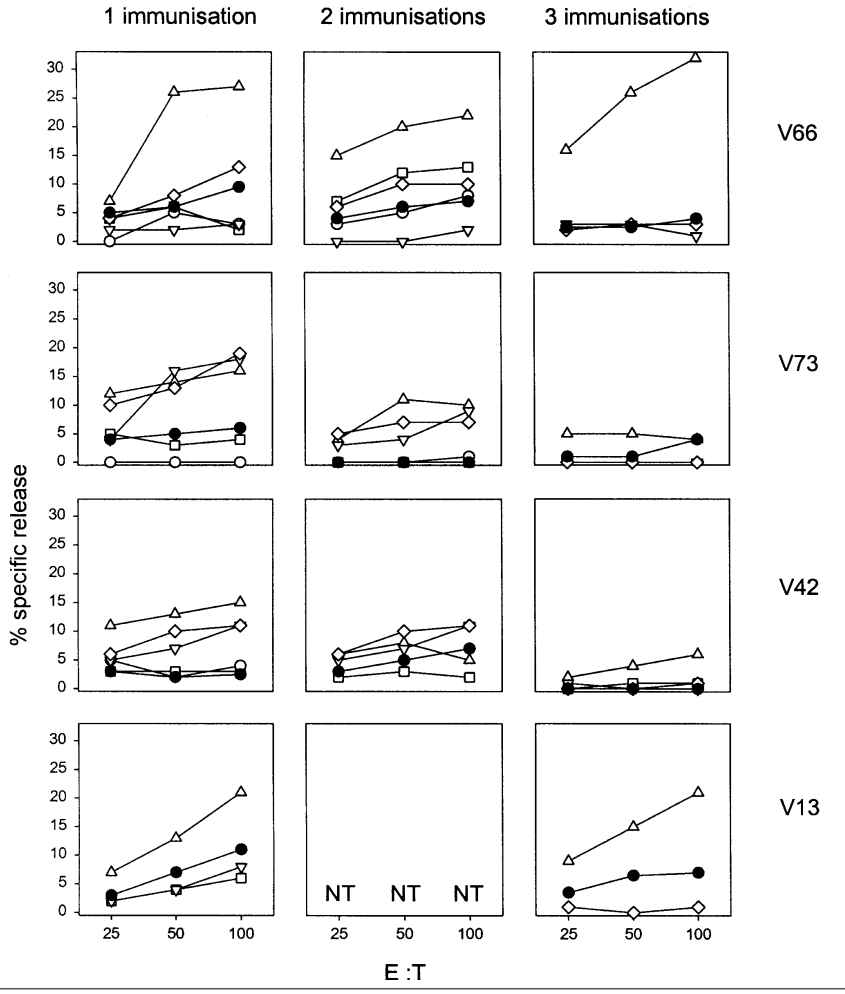

Fig. 1. Longitudinal analysis of SIV-specific CTL responses in four macaques induced by intramuscular immunization with MVA-SIVmacJ5 constructs. Responses were measured 4–8 weeks after the first immunization, 2 weeks after the second immunization and 2–6 weeks after the third immunization following mitogen-driven expansion of PBMC *in vitro*. Percent specific release of ^{51}Cr is shown for a range of effector to target ratios (E:T) on autologous B-LCL infected with recombinant vaccinia viruses expressing SIVmacJ5 gene products (▽, Rev; ◇, Tat; △, Nef; ○, Env; □, Gag–Pol) or influenza virus PB2 (●). NT, not tested.

multiplicity showed that only occasional cells had evidence of Gag expression revealed by particle formation and the characteristic accumulation of electron-dense material at the cell membrane (data not shown). Western blotting of infected cell lysates confirmed the synthesis of Gag protein, but only at a relatively low level (data not shown).

Anti-vector antibody responses following immunization with MVA-SIVmacJ5 constructs

Vaccinia virus (vector)-specific antibody responses were determined by ELISA at intervals throughout the immunization schedule. All animals made persistent humoral responses to the vaccinia virus vector throughout the immunization schedule. Notably, responses were induced by the initial vaccination and were boosted following the second vaccination but only poorly after the third (Fig. 3). Neutralizing antibody responses were weak and transient. In animals immunized with all of the MVA-SIVmacJ5 constructs, neutralizing antibodies were detected only after the second immunization (titres 19–46). By the time of the third vaccination, titres had dropped below the limit of detection. Similarly, following the third vaccination, a weak response (titres 18–24) was generated, declining rapidly (titres 4–9 by 4 weeks later). In animals 65T and 68T, similar low titres were detected (11 and 5 respectively, 9 weeks after the third immunization with *nef* and *gag–pol* constructs).

Induction of Gag-specific CTL in the presence of an anti-vector immune response

As described, immunization left both 65T and 68T with antibody responses specific for the MVA vector, but unresponsive with respect to detectable CTL directed against the Gag target antigen. Therefore, it was considered inappropriate to proceed at this stage to the analysis of local CTL immunity; however, these circumstances provided the opportunity to investigate the stimulation of CTL responses specific for the transgene in the face of anti-vector immunity. Hence, the animals were further immunized with a second construct; MVA-SIVsmH4 *gag–pol*, which had previously been shown to efficiently induce Gag epitope p11C C-M-specific CTL activity in naive Mamu A*01 rhesus macaques (Seth *et al.*, 1998).

Twenty-seven and 36 weeks after the last immunization with the MVA-SIVmacJ5 constructs animals 65T and 68T

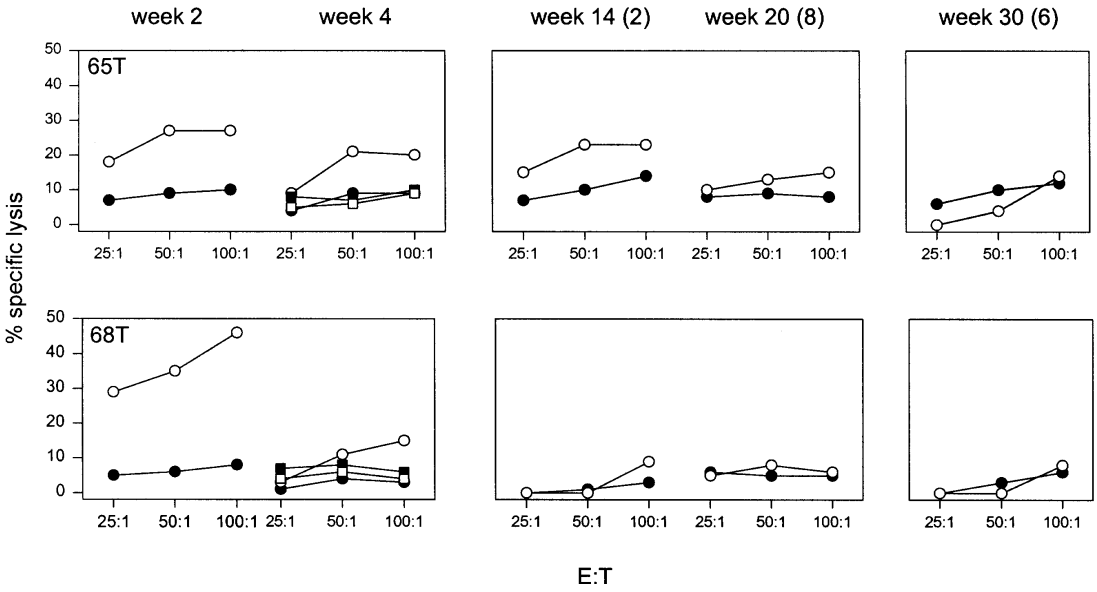

Fig. 2. Longitudinal analysis of Nef-specific CTL responses following intramuscular immunization of two macaques with MVA-SIVmacJ5 *nef* and MVA-SIVmacJ5 *gag–pol* constructs. Panels show results following each immunization; week refers to time after the first immunization and numbers in parentheses show weeks after the previous immunization. PBMC were expanded with mitogen *in vitro*. CTL activity is shown against autologous B-LCL targets infected with recombinant vaccinia virus expressing SIVmacJ5 *nef* (○) or influenza virus PB2 (●). MHC restriction of Nef-specific killing was determined at week 4 by additional incubation of effector cells with heterologous targets infected with recombinant vaccinia virus expressing SIVmacJ5 *nef* (□) or influenza virus PB2 (■).

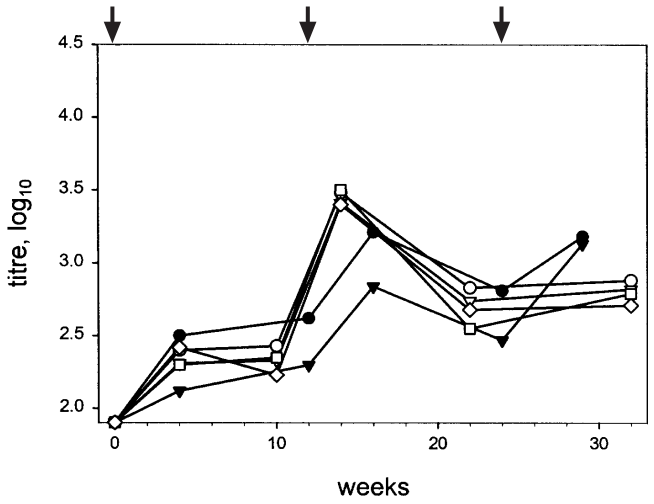

Fig. 3. Longitudinal analysis of antibody responses to the MVA vector following immunization with MVA constructs at the times indicated by the arrowheads. Responses are shown for animals immunized with five constructs on each occasion (○, V42; ▽, V73; □, V13; ◇, V66) and for animals immunized with two constructs (●, 65T; ▼, 68T).

were immunized intramuscularly with 10^8 p.f.u. MVA-SIVsmH4 *gag–pol*. A third animal, X19, a naive Mamu A*01$^+$ macaque, was included in this phase of the immunization schedule. PBMC from all three animals were assessed for CTL activity to the Mamu A*01-restricted Gag CTL epitope p11C C-M at regular intervals following immunization with the MVA-SIVsmH4 *gag–pol* construct (Fig. 4). Now, Gag-specific CTL activity was revealed in all three animals; however, the pattern of response induced in the previously naive animal, X19, was different to that seen in the animals with the pre-existing anti-vector humoral response (65T and 68T). CTL activity was first detected in X19 6 weeks after the initial immunization and persisted until the second boost at week 9. In 65T, only a weak and transient response was detected whilst in 68T there was an immediate large transient response. Notably, following further immunization, a response was only seen in animal X19.

Locally associated Gag-specific CTL responses induced by immunization with MVA-SIVsmH4 *gag–pol*

Gag-specific CTL responses were investigated in MNC isolated from systemic and mucosally associated lymphoid compartments. Following expansion *in vitro* with Gag p11C C-M peptide, CTL responses were revealed in the previously naive animal X19, in a wide range of lymphoid tissues (Fig. 4). In contrast, weak responses, restricted to inguinal and iliac lymph nodes, were seen in one animal that possessed pre-existing anti-vector immunity at the time of MVA-SIVsmH4 immunization (65T) and were not detected in any of the tissues analysed from the other (68T). No direct lytic activity, in the absence of expansion *in vitro*, was detected with freshly isolated cells taken from tissues of vaccinated animals.

In addition to the tissues shown in Fig. 4, *gag*-specific CTL were sought also in intraepithelial and lamina propria lymphocytes derived from the gut of each animal. Intraepithelial

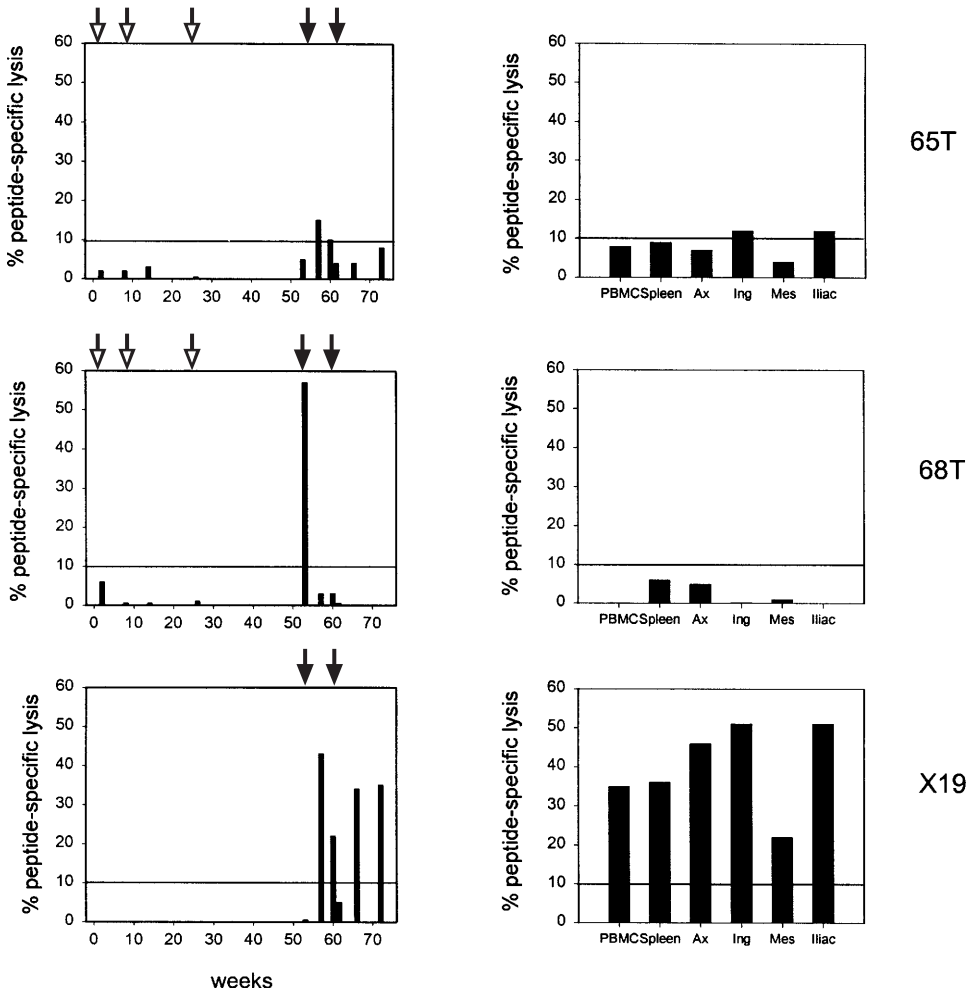

Fig. 4. SIV Gag-specific CTL responses induced in systemic and locally associated lymphoid tissues following immunization with MVA-SIVsmH4 *gag–pol* (⇓). Animals 65T and 68T were first immunized three times (⇓) with MVA constructs containing SIVmacJ5 *nef* and *gag–pol*. Gag-specific CTL activity was determined after restimulation *in vitro* with the Gag P11C C-M peptide and is shown for an E:T of 100:1. Left-hand panels show longitudinal activity in the peripheral circulation (PBMC). Right-hand panels show results in PBMC, spleen, axillary (Ax), inguinal (Ing), mesenteric (Mes) and iliac lymph nodes taken post-mortem at the time corresponding to the last data point shown in the left-hand panels.

lymphocytes were tested for direct CTL effector activity by chromium release assay immediately after isolation but none was detected, while lamina propria lymphocytes failed to survive the culture period *in vitro* with Gag p11C C-M peptide.

Discussion

Studies from several laboratories suggest that highly attenuated recombinant poxvirus vectors based on vaccinia virus may induce a degree of protective immunity in macaques against challenge with SIV. Hirsch *et al.* (1996) showed that virus loads were reduced following systemic challenge of macaques that had been vaccinated with MVA expressing SIV Env and Gag–Pol and boosted with inactivated virus. Another study using the vaccinia virus vector NYVAC expressing SIV structural genes has also given promising results and interestingly a high degree of protective efficacy was seen against rectal mucosal challenge with SIV despite the parenteral route of vaccination (Benson *et al.*, 1998). However, the correlates of protection in these studies were incompletely defined. Recently, immunization with MVA alone expressing SIV Gag–Pol and/or Env has been shown to suppress loads following challenge with pathogenic SIV (Ourmanov *et al.*, 2000).

In the study described here we were particularly interested in the generation of SIV-specific CTL responses as there is increasing evidence that CTL may have protective efficacy. Indeed, whilst this study was in progress, Seth *et al.* (2000) reported that the magnitude of reduction in viraemia in macaques vaccinated with MVA expressing SIV Gag–Pol was predicted by the magnitude of the vaccine-elicited CTL response prior to SIV challenge. In our study we vaccinated macaques with MVA constructs expressing not only structural but also regulatory/accessory genes of SIV. Rev, Tat and Nef

are known to be targets for CTL recognition and since they are expressed early in the virus replicative cycle CTL against them may clear virus-infected cells prior to the production of mature virions. We were encouraged that CTL responses to several SIV proteins were detected after only a single vaccination with MVA; however, in only two animals was an SIV-specific CTL response maintained after boosting, and then only to Nef. Furthermore, repeated immunization failed to reveal CTL responses to new specificities. The early detection of SIV-specific CTL is similar to the result reported by Seth *et al*., where 3/4 animals had CTL after a single vaccination; however, these workers found a significant boost of responses after the second immunization. Interestingly though, subsequent boosts had little or no effect (Seth *et al*., 1998, 2000). Furthermore, in these studies, the animal unresponsive after a single immunization generated CTL after the second immunization. Our study differed in several ways. Firstly, because of the number of antigens used, we employed a non-specific expansion method to reveal CTL activity: a method known to be relatively inefficient compared to antigen-driven restimulation (Kent *et al*., 1994; Voss *et al*., 1995). It is likely that mitogen restimulation reveals early CTL responses, when there is a large expansion of antigen-specific CTL *in vivo*. The apparent disappearance of responses at later times is therefore unlikely to be absolute; rather, it is likely due to an inability to expand a low frequency precursor pool. Why Nef-specific CTL responses remained high in V66 and V13 is unknown but interestingly these two animals were resistant to infection following rectal challenge with SIVmac251 (unpublished). Secondly, we used a 5-fold higher dose of virus for each immunization. Thirdly, with the exception of the *env* construct, a weaker promoter than that used by Seth *et al*. was employed to drive transgene expression.

The failure to detect Gag-specific CTL in PBMC from two Mamu A*01$^+$ vaccinates following peptide restimulation *in vitro* was surprising, especially so, as global restimulation with mitogen revealed MHC-restricted Nef-specific CTL. It is possible that immunization resulted in low numbers of Gag-specific CTL precursors that would be revealed only by more sensitive methods such as interferon-γ ELISPOT or prolonged expansion with several rounds of antigenic stimulation (McMichael & O'Callaghan, 1998; Vogel *et al*., 1998). The poor immunogenicity of the MVA SIVmacJ5 *gag–pol* construct was most likely due to the reduced levels of *gag–pol* gene expression found upon *in vitro* analysis of the large-scale expanded vaccine preparations.

Despite the restricted growth potential of MVA *in vivo*, anti-vaccinia virus antibodies were detected after a single immunization and were boosted subsequently upon further immunization. The antibody levels were, however, lower than those seen in a previous experiment in macaques using replication-competent vaccinia virus (Cranage *et al*., 1994). Here, titres of $\log_{10}$ 3·1 were still detectable 35 weeks after intradermal administration of two doses of 10^7 p.f.u. of recombinant WR strain vaccinia virus given 13 weeks apart. Studies in mice (Rooney *et al*., 1988; Belyakov *et al*., 1999) and in man (Cooney *et al*., 1991) show that pre-existing anti-vaccinia virus immunity can have a detrimental effect on vaccine efficacy when using replication-competent vaccinia virus vectors. Our data indicate that immunity to the MVA vector had an influence on the SIV Gag-specific CTL response generated following the immunization with a high level expression construct, MVA SIVsmH4 *gag–pol*. The responses seen in the naive animal were similar to those reported in an earlier study with this MVA construct (Seth *et al*., 1998), with Gag-specific CTL detected 6 weeks after immunization. The disappearance of responsiveness 10 days after the second boost is puzzling; however, responses subsequently recovered. This transient loss of an *in vitro* expandable response may be associated with vaccine-induced expansion *in vivo* of precursor CTL that can be detected by tetramer staining (Hanke *et al*., 1999; Seth *et al*., 1998). The rapid, high response detected in 68T suggests that this animal had been primed by the exposure to MVA-SIVmacJ5.

In murine studies, Belyakov *et al*. (1999) showed that pre-immunization with the WR strain of recombinant vaccinia virus prevented the generation of a CTL response and reduced antibody titres to HIV-1 gp160 following immunization with an MVA construct expressing this antigen. Recently it was shown, also in mice, that MVA can be used as an immunizing agent under conditions of pre-existing immunity induced by MVA (Ramirez *et al*., 2000). Nevertheless, there was a considerable reduction in the number of interferon-γ-secreting cells specific for the transgene induced in the presence of pre-existing immunity, albeit this reduction was less than that seen following prior immunization with vaccinia virus WR strain. These findings are consistent with those reported here in primates. Furthermore, the relatively large dose of MVA used in the present study may have favoured the generation of strong anti-vector immunity.

The mechanism by which pre-existing anti-vector immunity limits response to delivered antigens is not known and may involve both humoral and cellular immunity. Anti-vector antibody may reduce the take of vaccine doses through neutralization of the inoculum, although in the present study such responses were weak and short lived, or by promotion of clearance of factory sites through mechanisms such as antibody-dependent cellular cytotoxicity. Likewise, an MVA-specific CTL response, not measured in the current study, may limit antigen production.

Although limited to observations in one animal, which had not previously been exposed to MVA, we were encouraged by the disseminated CTL response detected. The presence of CTL reactivity in cells from the iliac lymph nodes was of particular interest. These nodes drain the recto-genital tract and represent an important site of SIV dissemination following both vaginal (Miller *et al*., 1992; Spira *et al*., 1996) and rectal (Lehner *et al*., 1996) infection of rhesus macaques. Furthermore, immune

responses elicited by targeting the iliac lymph nodes have been associated with protection against rectal mucosal challenge (Lehner *et al.*, 1996). The failure to detect direct CTL activity in intra-epithelial lymphocytes, suggests that mucosal immunity per se was not stimulated by parenteral vaccination with MVA, although we cannot exclude the possibility that precursor CTL were present. This is in agreement with mouse data showing that systemic immunization with MVA failed to elicit mucosal immunity (Belyakov *et al.*, 1998).

Overall, our results and those of others suggest that MVA stimulates both a circulating and disseminated CTL responses to SIV transgenes, but the efficiency of stimulating strong and persistent responses is critically dependent upon the level of anti-vector immunity generated and the efficiency of transgene expression from the vector. Further work is required to define optimal immunization protocols, including those using mixed vector or DNA priming steps that may focus the immune response away from the vector and onto the transgene.

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