Recombinant vaccinia viruses for the characterization of Plasmodium falciparum-specific cytotoxic T lymphocytes: recognition of processed antigen despite limited re-stimulation efficacy

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Abstract

Cytotoxic T lymphocytes (CTL) have been implicated in immunity to Plasmodium falciparum infection and disease. We have previously described the use of peptides to define malaria-specific CTL epitopes. To determine whether these peptide epitopes are processed intracellularly from the whole antigen we have developed recombinant vaccinia viruses (rVV) expressing three malaria antigens: thrombospondin-related adhesive protein (TRAP), Pfs16 and the C-terminal half of liver-stage antigen (LSA)-1. Target cells infected with recombinant viruses were lysed by malaria-specific CTL from semi-immune African donors. We also tested the ability of cells infected with these recombinant vaccinia viruses to re-stimulate malaria-specific CTL in peripheral blood lymphocytes from malaria immune adults. Two other pox virus recombinants, NYVAC, an attenuated vaccinia virus, and ALVAC, a canarypox virus, both expressing malaria antigens were also evaluated for their ability to stimulate malaria-specific CTL. In contrast to peptide, none of these viruses successfully re-stimulated CTL from the peripheral blood lymphocytes of semi-immune donors. The ability of human CTL from naturally exposed individuals to recognize processed antigen supports the relevance of these cells in protective immunity to malaria.

Introduction

Class I-restricted CD8+ cytotoxic T lymphocyte (CTL) responses have been shown to be a critical arm of the protective immune response to intracellular pathogens and in some rodent models of malaria the presence of CD8+ CTL has been shown to correlate with protection from infection (1–3). In Plasmodium falciparum infections of humans the evidence that CTL may play a protective role is indirect. The pattern of sequence variation in certain CTL epitopes (4,5), the finding of an HLA class I association with resistance to severe malaria (6) and the identification of malaria-specific CTL in naturally exposed as well as sporozoite-immunized individuals all suggest that CTL may play some protective role (7–12).

Human CTL were originally defined to viral pathogens (13) where the use of the virus in in vitro cultures provides a convenient method of re-stimulating specific CTL. However, this option is not available in studies of malaria CTL as it is not possible to culture the liver-stage parasite except in a very low percentage of freshly isolated hepatocytes. Thus, the convincing identification of CTL against P. falciparum awaited the development of peptide-based re-stimulation strategies (9) which have recently been developed extensively (10). However, it would be beneficial if whole antigen systems could be employed for human CTL analysis for two reasons. Firstly, this would overcome the need to use peptides appropriate for particular
HLA molecules to detect CTL and allow an overall measurement of the level of the CTL responses to a malaria antigen to be made, independent of the HLA type of the individual. Secondly, this would allow assessment of whether human CTL to \( P. falciparum \) are capable of recognizing endogenous processed antigen as well as peptide epitopes applied externally to the surface of target cells. Clearly, to play a protective role, CTL should be able to recognize processed antigen as well as peptides.

To address these issues, we have constructed recombinant vaccinia viruses (rVV) containing three pre-erythrocytic antigens of malaria, two of which we have found to be major CTL-inducing antigens, liver-stage antigen (LSA)-1 and thrombospondin-related adhesive protein (TRAP) (10). Additionally we have explored the utility in CTL re-stimulation of canarypox (ALVAC) and attenuated vaccinia virus (NYVAC) recombinants containing LSA-1. We find that CTL from naturally exposed individuals are capable of lysing target cells infected with these recombinant viruses, but that the ability of these recombinants to re-stimulate CTL in \textit{in vitro} culture is less than that of short synthetic peptides.

**Methods**

**Viruses, cells and parasites**

Vaccinia virus (strain WR) was propagated in baby hamster kidney (BHK) cells as described (15) and recombinant viruses in thymidine kinase-negative cells (TK–143) or, for larger quantities, in BHK cells. BHK cells were grown in Glasgow modified Eagle’s medium with tryptose phosphate broth supplemented with 10% newborn calf serum. TK–143 cells were grown in Dulbecco’s modified Eagle’s medium with sodium pyruvate and 1 g/l glucose supplemented with 10% FCS. \( P. falciparum \) (clone T9/96), a gift from Professor David Walliker, was cultured as described (16) and DNA extracted as described by Robson and Jennings (17).

**Plasmid construction**

Oligonucleotide primers were designed to enable the complete coding sequences of the genes for Pf16 and TRAP and partial sequences of LSA-1 and TRAP to be amplified using the PCR. The primers and the sequences amplified and the cycling conditions are shown in Table 1. The target DNA was from the cloned line of \( P. falciparum \), T9/96. PCR products were prepared for blunt end cloning into the \textit{Smal} site of the plasmid pSC11 (18) as described (19). Recombinants were detected by colony hybridization using end-labelled oligonucleotides internal to the PCR primers. The authenticity and orientation of the inserts were confirmed by restriction enzyme analysis and dideoxy-nucleotide sequencing using modified T7 DNA polymerase (Sequenase; US Biochemicals, Les Ulis, France) (20).

**Recombinant viruses**

Recombinant plasmids were purified by CsCl density gradient centrifugation. TK–143 cells infected with vaccinia virus (strain WR) at 0.05 p.f.u./cell were transfected with the appropriate calcium phosphate-precipitated plasmid DNA (15). TK–143 recombinants were isolated by plaque assay on human TK–143 cells in the presence of 5-bromodeoxyuridine and by their ability to express \( \beta \)-galactosidase. Recombinant viruses were purified by plaque purification and DNA isolated as described (15). The presence of the appropriate inserts was confirmed by PCR. The circumsporozoite protein (CSP) rVV was a gift from B. Moss (NIH, Bethesda, MD) (21). ALVAC expressing LSA-1 alone or CSP alone and NYVAC expressing LSA-1 alone or CSP alone were a gift from Virogenetics (Troy, NY). The LSA-1 gene insert used in these recombinant viruses was without the central repeat region.

**Protein analysis**

TK–143 monolayers were infected at high multiplicity of infection (30 p.f.u./cell) with wild-type virus (WR) and each of the recombinant viruses for 2 h, cells were rinsed, and the infection allowed to continue in complete medium. Cells were harvested 16 h post-infection and lysates prepared using the method described by Mackett et al. (15). Both lysates and culture supernatants were examined by Western blot analysis for the presence of the appropriate recombinant protein. Polyacrylamide gels varying from 7.5 to 12.5% were blotted onto polyvinylidene difluoride membranes and recombinant proteins detected using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). TRAP constructs were detected using a sheep anti-TRAP polyclonal antisera. LSA-1 was detected using rabbit antipeptide antisera, a gift from M. R. Hollingdale. Pf16 was detected using a rabbit antisera raised against recombinant Pf16 containing a histidine tag at its C-terminal end, a gift from R. Konings.

**Subjects**

Peripheral blood lymphocytes (PBL) from healthy Gambian adults were separated from heparinized whole blood by Ficoll gradient centrifugation and washed three times with R0 (RPMI 1640; Sigma, Poole, UK) supplemented with 100 U/ml penicillin, 100 \( \mu \text{g/ml} \) streptomycin and 4 mM \( \text{l}-\text{glutamine} \).

**Peptides**

Peptides were synthesized based on F-moc chemistry by an automated peptide synthesizer (Zinsser Analytic). All peptides were confirmed to be >70% pure by HPLC analysis and dissolved in PBS or first with 10–20 \( \mu \text{l} \) DMSO and subsequently diluted with PBS to bring the concentration of DMSO to <1%.

**CTL re-stimulation**

For peptide re-stimulation, 1.5–2\( \times \)10^7 PBL from malaria immune donors were incubated with 20 \( \mu \text{M} \) peptide in 100 \( \mu \text{l} \) of R10 (RO with 10% FCS) for 1 h at 37°C in the presence of 5% \( \text{CO}_2 \) and humidity. Cells were then diluted to a concentration of about 1–1.5\( \times \)10^5/ml in R10 and plated out in 2 ml/well in 24-well tissue culture plates (Falcon). Plates were incubated in a humidified incubator at 37°C in the presence 5% \( \text{CO}_2 \). After 72 h, 10 \( \mu \text{l} \) of human rIL-2 (Cetus, Emeryville, CA) was added to the cultures and cytotoxic assays performed at day 8 as described (10). Cells not used were maintained on a weekly dose of 10 \( \mu \text{l} \) IL-2 until tested for cytotoxicity. For CTL re-stimulation with recombinant virus, 20% of PBL from a malaria immune donor were infected with 5–20 p.f.u. of virus/cell and used as stimulator cells. When paraformaldehyde fixation was used, overnight cultures of virally infected
cells were resuspended in 5 ml of 1.5% w/v paraformaldehyde and incubated at 37°C for 20 min. The cells were washed and 5 ml of 0.2 M glycine added for 20 min at 37°C followed by two washes. Virus-infected stimulator cells were incubated with the rest (80%) of the PBL in 24-well plates at 37°C and 5% CO2 in a humidified incubator. Recombinant virus cultures were incubated with rIL-2 from 72 h onwards for wild-type vaccinia recombinants but not for ALVAC or NYVAC recombinants according to previous optimization studies.

**Target cells and cytotoxicity assays**

Target cells were Epstein–Barr virus-transformed autologous or HLA-matched B lymphoblastoid cell lines. Then 1–2×10^6 target cells were radioactively labelled with 100 μCi of ^51^Cr for 1 h at 37°C, washed once and in the case of peptide-pulsed targets washed with 10–20 μM of peptide for a further 1 h. After washing, the cells were diluted in R10 to 10^5/ml and plated out 50 μl/well in 96-well round bottom tissue culture plates. For virus-infected target cells, cells were infected with 1 h with 5–20 multiplicity of infection (m.o.i.) of either recombinant virus of interest or an irrelevant recombinant virus in a small volume (100 μl) of R10 at 37°C. The irrelevant vaccinia was used as a control because many of the subjects studied were likely vaccinated some 10–30 years ago. Cells were washed once, diluted to R10 to ~1×10^6/ml and incubated overnight at 37°C in 5% CO2. Radiolabelling of virus-infected targets was as for peptide-pulsed targets. ^51^Cr-release cytotoxicity assays were performed as described (9). As usual, lysis assays were performed in duplicate wells and the mean calculated. A positive result was defined as 15% specific lysis of target cells pulsed with relevant peptide or infected with relevant recombinant virus over targets not pulsed with peptide or infected with irrelevant recombinant virus: the percentage lysis presented in the figures represents this difference. Spontaneous ^51^Cr release in the absence of CTL was always <25% of maximum release by 5% Triton.

**Results**

**Construction of rVV**

The amplified gene sequences were cloned into the Smal site of pSC11 which is downstream of the p7.5 promoter and flanked by TK sequences. The plasmids generated were pSC11TRAP, pSC11Pfs16, pSC11LSA-1/8.5 and pSC11CRAP15. Sequencing of the inserts of these plasmids confirmed their authenticity and whether any errors had been incorporated during PCR amplification. One error had occurred in the CRAP15 construct, slippage in a block of poly(dA) towards the end of the sequence caused a change in reading frame such that the last 12 amino acids after the three in-frame lysines were ENQIINIKLQVE giving a final length of 500 amino acids where 488 corresponded to the authentic T9/96 TRAP sequence. These plasmids were used to co-transfect TK−143 cells with vaccinia virus (WR), TK−indicated that the required sequences had indeed been inserted into the TK gene of the relevant vaccinia virus. Recombinant proteins synthesized by the recombinant viruses were detected by Western blot analysis. Both of the recombinant TRAP constructs were found both in the virus lysates and in the culture supernatants. This was not an unexpected finding as TRAP expressed in recombinant baculovirus behaves in the same way (22). The vvCRAP15 construct gave a product of ~70 kDa which is comparable to the bacterially expressed product of TRAP1.0 (22) which encodes amino acids 26–498 of TRAP. The full-length construct vvTRAP gave a product of similar size and can be compared to the product expressed in recombinant baculovirus and this may represent processing of the full-length protein in these systems (Fig. 1).

Pfs16 gave a protein of expected size on Western blots. It was only found in the cell lysate fraction as recently reported for an independently constructed recombinant virus (23). The C-terminal portion of LSA-1 that was expressed had been predicted to produce a protein of ~30 kDa. Both antipeptide sera detected two closely migrating bands of much larger mol. wt. ~45 kDa. Although this was an unexpected finding, many malarial proteins migrate anomalously in denaturing polyacrylamide gels and this observation for LSA-1 has been confirmed by M. Hollingdale (pers. commun.). It is possible that the decreased mobility is due to glycosylation. There is one N-linked glycosylation consensus sequence in this part of the molecule. Alternatively it could be due to some other post-translational modification. LSA-1 protein was found in the cell lysates and some was also detected in the culture supernatants.

**Peptide-re-stimulated CTL recognize processed antigen**

We tested the ability of LSA-Vac-infected target cells to process and present malaria peptides to synthetic peptide re-stimulated CTL. CTL lines specific for HLA-B53-restricted CTL epitope ls6 (9) recognized LSA-Vac-infected target cells
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Fig. 2. Peptide re-stimulated CTL recognize peptide on the target cell surface after endogenous processing of recombinant antigen. Ls6 re-stimulated CTL lysed Ls6-pulsed target cells and vaccinia-LSA-1 (LSA-Vac)-infected cells but not vaccinia-HIV-gag (HIV-gag-Vac)-infected cells. The effector:target ratio for all assays was 50:1.

Fig. 3. PBL from a malaria immune individual with HLA-B53 who responds to Ls6 were stimulated either with Ls6 peptide or LSA-Vac-re-stimulated CTL lysed Ls6-pulsed target cells and vaccinia-infected autologous PBL. Whereas peptide-stimulated cells lysed Ls6 pulsed targets there was no lysis of these targets by LSA-Vac re-stimulated cells. The effector:target ratio for both assays was 50:1.

although the level of lysis was not as high for peptide pulsed targets (Fig. 2). HIV-gag-Vac-infected targets were not recognized by these CTL. This result showed that Ls6 was processed endogenously from the Lsa-Vac antigen and was recognized by Ls6-specific CTL. Recently, in studies of malaria-immune Tanzanians we have found that CTL specific for the peptide epitopes tr26, tr29 and tr39 from TRAP recognize target cells infected with the recombinant virus (24). To date we have not detected CTL to Pf16 in any malaria immune donor, so it has not been possible to test for lysis of target cells infected with the rVV virus.

CTL re-stimulation with rVV expressing malaria antigens

Two major requirements for the use of recombinant virus-infected stimulator cells for in vitro CTL stimulation are that there should be a way of limiting reproductive replication of the virus so that lysis of the antigen-presenting cell by the virus does not occur and also that there is early and adequate expression of the inserted antigen. The former may be achieved by UV irradiation of virus-infected cells after a few hours post-infection in vitro (25). However, preliminary studies of this inactivation method failed to yield effective CTL re-stimulation. Other methods used are paraformaldehyde fixing (26) or mitomycin treatment of virus-infected stimulator cells. Here we have used paraformaldehyde fixation which has been previously reported to successfully re-stimulate HIV-specific CTL (26).

We failed to demonstrate re-stimulation of malaria-specific CTL with rVV expressing LSA-1 (Fig. 3), CSP, Pf16 and TRAP after testing on peptide pulsed targets and rVV-infected targets (data not shown). In some assays where rVV infected targets were used, there was enhanced lysis of wild-type vaccinia-infected targets, targets infected with rVV expressing P. falciparum antigens and targets expressing HIV-gag. Such non-specific lysis of wild-type vaccinia virus and rVV has been reported previously (27).

CTL re-stimulation with other recombinant viruses

Recently highly attenuated viruses and viruses with restricted growth in certain host cells have been described (28,29). Two such recombinant virus delivery systems are being developed by Virogenetics. One is NYVAC, an attenuated vaccinia virus characterized by a lack of 18 open reading frames resulting in decreased virulence and altered infectivity of certain host cells (30). The other is ALVAC, a canarypox virus vector which replicates productively only in avian cells, and has been shown to process and present foreign gene inserts in mammalian cells (31). Both viruses are non-pathogenic in man.

From a malaria-immune adult who responds to a nonamer peptide from the P. falciparum liver-stage antigen LSA-1, Ls6, peptide-re-stimulated CTL from PBL lysed targets pulsed with Ls6 in a 4 h Cr-release assay (Fig. 4). These CTL were Ls6 specific and did not lyse target cells pulsed with irrelevant control nonamer epitopes from CSP, cp6, or LSA-1, Ls23; a peptide that does not bind to HLA-B53 in binding assays (data not shown). Cells from the same individual taken at the same time but re-stimulated with autologous PBL infected with NYVAC-LSA 1, ALVAC-LSA 1 and LSA-Vac, all recombinant viruses, showed no lysis of Ls6-pulsed target cells (Fig. 3). This result showed that although it was possible to re-stimulate Ls6-specific CTL with peptide and show lysis of peptide-pulsed targets, lysis of these target cells was not observed following re-stimulation with recombinant virus-infected donor PBL. It has been suggested (27) that a low m.o.i. leads to a preferential response of T cells to the foreign gene insert and not the vector, but for ALVAC-LSA neither m.o.i. of 5 or 20 re-stimulated detectable CTL as assayed by lysis either of peptide-pulsed targets (Fig. 4) or LSA-Vac-infected targets (not shown).

Discussion

The low precursor frequencies of CTL to P. falciparum in naturally exposed individuals (10 and Plebanski et al., submit-
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Ld-positive cells using peptides known to be restricted by H-2L^d. Also, it has been shown that there is down-regulation of HLA class I molecule expression on the cell surfaces after viral infection (34–36). These two phenomena occurring on peptide-pulsed and virus-infected target cells would lead to a marked difference in the levels of class I MHC expressed on the cell surfaces. In situations where down-regulation of virus-infected cells significantly reduces cell surface class I expression, there may be less than optimal peptide presentation resulting in an impaired ability of these cells to re-stimulate CTL. As we have shown here, even when virus-infected targets are lysed by CTL, the level of lysis is lower than lysis of peptide-pulsed targets by the same CTL. It has been suggested that CTL re-stimulation in vitro requires the presence of more peptide–MHC complexes on the cell surface than is required for sensitization of targets for lysis by CTL (33). Alternatively, we speculate that infection of dendritic cells by vaccinia viruses in vitro may be inefficient. Thus, if dendritic cells are important for in vitro re-stimulation, loading of dendritic cell class I molecules might be achieved more efficiently by external peptide than by these viruses. An additional difficulty with the use of rVV is the technical issue of dealing with vaccinia virus-specific CTL and possibly other killer cells not specific to the recombinant antigen. Most of our Gambian population studied here have been vaccinated and we occasionally found high levels of lysis of wild-type vaccinia-infected target cells.

The development of the ALVAC and NYVAC recombinant viruses is a promising advance for human vaccine development. However, we failed to show in vitro CTL stimulation of PBL from individuals naturally exposed to malaria despite encouraging evidence of the immunogenicity of other such recombinants in mice and humans. ALVAC and NYVAC carrying the HIV-1 env gene have been shown (14) to elicit protective immunity in naive mice and this immunity is mediated through both antibody and CTL. A P. berghei NYVAC-CSP recombinant has been found to produce significant protection against this murine malaria species (37). NYVAC-P7, a rVV expressing seven P. falciparum antigens, is currently more efficient than re-stimulation by wild-type or attenuated canarypox virus (ALVAC). We were not able to compare these re-stimulation protocols with stimulation with transfected cells expressing relevant antigens or proteins. However, studies of hepatitis CTL comparing PBL loaded with peptide to transfected autologous cells found that HBV-specific CTL were consistently detected only by peptide re-stimulation (32). Van Baalen et al. (26) have reported the detection of HIV-specific CTL employing re-stimulation with paraformaldehyde-fixed recombinant vaccinia-infected cells as employed here. However, HIV-specific CTL precursor frequencies are often much higher than these observed to malaria epitopes, suggesting that, in the presence of a higher precursor frequency of CTL, such a method may be effective.

However, our data strongly suggest that short peptides are more efficient re-stimulators of malaria CTL than recombinant viruses. The explanation for this difference is uncertain. However, peptides can up-regulate the expression of class I molecules on some cells. Alexander and co-workers (33) reported a 2- to 4-fold increase in the level of L^d on cultured L^d-positive cells using peptides known to be restricted by H-2L^d. Also, it has been shown that there is down-regulation of HLA class I molecule expression on the cell surfaces after viral infection (34–36). These two phenomena occurring on peptide-pulsed and virus-infected target cells would lead to a marked difference in the levels of class I MHC expressed on the cell surfaces. In situations where down-regulation of virus-infected cells significantly reduces cell surface class I expression, there may be less than optimal peptide presentation resulting in an impaired ability of these cells to re-stimulate CTL.
been demonstrated in some rodent models of plasmodial infection (39).

The observation that CTL epitopes identified by peptide re-stimulation in naturally exposed individuals are correctly processed when presented by recombinant viruses encourages further development of these peptides as constituents of future CTL-inducing vaccines.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
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<tr>
<td>CSP</td>
<td>circumsporozoite protein</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>LSA</td>
<td>liver-stage antigen</td>
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<tr>
<td>m.o.i.</td>
<td>multiplicity of infection</td>
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<tr>
<td>PBL</td>
<td>peripheral blood lymphocyte</td>
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<tr>
<td>rVV</td>
<td>recombinant vaccinia virus</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
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<td>TRAP</td>
<td>thrombospondin-related adhesive protein</td>
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References


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