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Induction of cytolytic T lymphocytes directed towards the V3 loop of the human immunodeficiency virus type 1 external glycoprotein gp120 by p55^{gag}/V3 chimeric vaccinia viruses

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T cell-mediated cytotoxicity may play an important role in controlling infection by human immunodeficiency virus (HIV). In order to study the ability of rationally designed antigens to induce cytolytic T lymphocytes (CTLs) we replaced stretches of 30 to 50 amino acids at the p17-MA/p24-CA cleavage site, within the p24-CA moiety and within the p6-LI portion of the HIV type 1 p55^{gag} precursor by the third variable domain (V3) of the external glycoprotein gp120. This site is known to be a target for CTL attack in mice and humans. The chimeric antigens were recombined into highly attenuated vaccinia viruses in order to investigate class I major histocompatibility complex (MHC)-restricted presentation of antigenic V3 peptides. Immunoprecipitation and Western blot analysis of the group-specific antigen (p55^{gag})/V3 chimeric proteins demonstrated significant differences in the accessibility of the V3 domain for a monoclonal antibody or polyclonal V3-specific antisera, depending on the position of the V3 loop within the p55^{gag} carrier protein. Immunization of BALB/c mice with three variants of p55^{gag}/V3 recombinant vaccinia virus, however, resulted in a comparable priming of CD4$^-$ CD8$^+$ CTLs *in vivo* irrelevant of the position of the V3 loop within p55^{gag}. Local conformational changes, including the V3 domain within the p55^{gag}/V3 chimeras, did not demonstrate a significant effect on V3-specific lysis of the target cells when compared to the authentic gp120 envelope protein. Class I MHC-restricted CTLs induced by a V3 consensus sequence cross-reacted perfectly with the LAI strain-derived V3 loop sequence. These data indicate that the combination of selected epitopes (V3) with immunologically relevant complex carrier proteins (p55^{gag}) can be accomplished without the loss of biological activity.

Introduction

Most successful vaccines closely mimic the pathogen or the natural infection, implying that natural infection leads to a long-lasting immunity. This is not so for human immunodeficiency virus (HIV) infection in humans. There is evidence that certain species of antibodies directed at defined epitopes within the HIV type 1 envelope proteins gp120 and gp41 can enhance the ability of HIV to infect macrophages and monocytes (Robinson *et al.*, 1989, 1990; Takeda *et al.*, 1988) and contribute to severe immune dysfunctions by cross-reacting with modulators of the immune response such as class II major histocompatibility complex (MHC) molecules (Lasky *et al.*, 1987; Young, 1988; Golding *et al.*, 1988, 1989) and certain IgA and IgG subclasses (Maddon *et al.*, 1986; Bjork, 1991). Most of these adverse effects are associated with the envelope glycoproteins gp41 and gp120, which can directly contribute to the physical and functional elimination of helper T cells by binding to the CD4 receptor, thus labelling these cells for immune attack (Weinhold *et al.*, 1989; Siliciano *et al.*, 1988).

With respect to future vaccine development, rationally designed antigens should be as complex as possible. For safety reasons, however, epitopes known to be associated with negative side-effects should be excluded and only immunologically defined epitopes, involved in eliciting protective immune responses, should be considered for a candidate vaccine. To allow favourable presentation of these epitopes to the immune system we developed a particulate, non-infectious and autologous carrier system based on the HIV-1 p55 group-specific antigen (p55^{gag}; Wagner *et al.*, 1991). By introducing foreign sequences into the native p55^{gag} protein, relevant epitopes can be presented by these virus-like particles (R. Wagner *et al.*, unpublished). In addition to their crucial role during the budding process (Göttlinger *et al.*, 1989; Wagner *et al.*, 1992*a*: von Poblotzki *et al.*, 1993) the HIV core proteins are able to induce inhibitory antibodies (Papsidero *et al.*,

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1989; Wolf *et al.*, 1990) as well as cytolytic T lymphocytes (CTLs) (Nixon *et al.*, 1988; Phillips *et al.*, 1991). This suggests that p55^{gag} is an appropriate, immunologically relevant carrier component.

Epitopes to be inserted into this carrier protein should induce both neutralizing antibodies and an effective cellular immune response. This has been demonstrated for the third variable domain (V3) of HIV-1 gp120 (Rusche *et al.*, 1988; Palker *et al.*, 1988; Goudsmit *et al.*, 1988; Clerici *et al.*, 1991). The induction of CTLs by a vaccine is of particular interest because of observations by several groups (Walker *et al.*, 1987, 1989; Tsubota *et al.*, 1989) that CD8$^+$ CTLs could prevent outgrowth of HIV *in vitro*. If CTLs do the same *in vivo* (as suggested by D. Mosier at the international conference on AIDS), stimulation of this part of the immune response might prove very helpful in controlling disease. The ability of the V3 domain to generate CTLs, not only in humans but in BALB/c mice (Takahashi *et al.*, 1988, 1990*a*, *b*, 1992; Hart *et al.*, 1991), allows a rapid evaluation of chimeric antigens in a convenient animal model, and therefore suggests the V3 domain as a candidate epitope for insertion into the p55^{gag} carrier protein.

One critical aspect in the induction of class I-restricted CD8$^+$ CTLs by chimeric antigens is whether altered flanking sequences or changes in the local conformation at an antigenic site might co-determine processing and presentation of a translocated epitope. In order to address this question, three regions within the p55^{gag} precursor, the MA/CA cleavage site and the regions within the p24-CA domain product and the p6 portion of p55^{gag}, were selected for their predicted logarithmic surface probabilities (Modrow *et al.*, 1987) and replaced by a consensus sequence of the V3 domain (V3c). This epitope, previously designed to overcome the isolate specificity of antibodies directed towards the V3 domain, was shown to mediate killing of target cells by CD4$^-$ CD8$^+$ CTLs induced in mice by a gp120 (HIV strain LAI; gp120$_{\mathrm{LAI}}$) recombinant vaccinia virus (VV) (Wagner *et al.*, 1992*b*). Immunization of BALB/c mice with recombinant VVs expressing different p55^{gag}/V3c chimeric antigens should provide more information about the induction of CTLs by such artificial antigens.

Methods

Mice. BALB/c mice (H-2^d haplotype, Jackson Laboratories) were bred under specific pathogen-free conditions.

Cells. CV-1 cells and 143B cells, grown in DMEM plus 10% fetal calf serum (FCS) (Gibco) were used to establish recombinant VVs. P815 cells, a continuously growing mastocytoma cell line (in RPMI with 10% FCS; Gibco), was used for the *in vitro* stimulation of splenocytes and as target cells in a 3 h cytolytic assay.

Monoclonal antibodies. The monoclonal antibodies (MAbs) anti-Lyt2 (3.155; rat IgM) (Sarmiento *et al.*, 1980) and anti-L3T4 (RL172.4; rat IgM) (Ceredig *et al.*, 1985) were used to characterize the surface phenotype of the effector cells. The V3-specific murine MAb, Nea 9301 (Du Pont), recognizes a central motif of the V3 loop region (RIQRGPGRAFVTIGKI), and the p24-CA-specific MAb used (16/4/2) was previously mapped to amino acids 307 to 336 within the capsid moiety of the p55^{gag} precursor (Wolf *et al.*, 1990).

Selection of deleted epitopes. The three regions within the p55^{gag} precursor, located at the MA/CA cleavage site, within the p24-CA domain product and within the p6-LI portion of p55^{gag}, were selected for their predicted antigenic indices and their logarithmic surface probabilities using computer-assisted secondary structure analysis of the HIV-1 group-specific antigen (UWGCG software).

Construction of chimeric antigens. A new pUC8 derivative, plin8, has been constructed (Wagner *et al.*, 1992*a*) and was treated as follows. The pUC8 multiple cloning site was replaced by a synthetic linker sequence containing all restriction sites (*Bam*HI, *Hin*dIII, *Xho*I, *Sac*I, *Pst*I, *Spe*I and *Sal*I) necessary for the construction of the described plasmids (Fig. 1). In order to delete the p17-MA/p24-CA cleavage site, including flanking amino acids 99 to 154, a 300 bp *Bam*HI/*Hin*dIII fragment encoding the terminal part of p55 was cloned into the *Bam*HI/*Hin*dIII site of plin8 (plin8p55BH). The 1283 bp *Nsi*I/*Sal*I fragment encoding the C-terminal part of p55^{gag} was inserted into the *Pst*I/*Sal*I site of plin8p55BH to form plin8p55Δ1. A stretch of 30 amino acids (311 to 341) located within the p24-CA moiety was deleted by subcloning the complete 1752 bp *Bam*HI/*Sal*I fragment of pUC8p55 into the *Bam*HI/*Sal*I sites of plin8 (plin8p55) and replacing a 90 bp *Pst*I/*Spe*I fragment by a 27 bp *Xho*I/*Sac*I linker fragment (TGCAGCTCGAGAATTCGAGCTCACTAG) (plin8p55Δ2). For the construction of deletion mutant p55Δ3 (lacking amino acids 483 to 471 within p6), the original 3′ part of the p55^{gag} coding sequence was replaced by a PCR fragment, amplified using a 64 bp 5′ primer (CGACTCGGATCCAAGATCTCTCTCGAGAATTCGAGCTCG AAGAGAGATTCAGGTCTGGGGTAGA) containing four 5′ restriction sites (*Bgl*II, *Xho*I, *Eco*RI, *Sac*I) and a 21 bp 3′ primer (TTCCAATTATGTCGACAGGTG) containing a 3′ *Sal*I site. The amplified *Bgl*II/*Sal*I fragment was inserted into the corresponding vector fragment of plin8p55 (plin8p55Δ3). The V3c sequence was then inserted into the *Xho*I/*Sac*I sites of the above described p55 deletion constructs to create the p55^{gag}/V3c-1, -2, and -3 chimeric genes (Fig. 1). All subcloned V3 sequences including the flanking regions were verified by dsDNA sequencing.

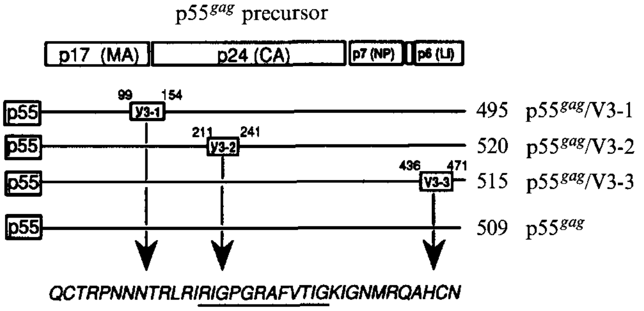

Fig. 1. Schematic drawing of three different p55V3c constructs. The location of the V3c sequence relative to the p55^{gag} precursor is marked with the amino acid positions of the deleted gag sequences. The total number of amino acids of the resulting polypeptides is indicated. The designation of the resulting chimeric VVs is given at the right side of the figure. The V3 domain that replaced each of the gag sequences is indicated below the constructs and the 12-mer within the 36-mer consensus peptide, an optimal CTL antigenic site, is underlined.

Recombinant vaccinia viruses. Recombinant VVs were established according to standard procedures (Mackett *et al.*, 1984) after subcloning of the *Bam*HI/*Sal*I fragments into the pAvB VV transfer vector (von Brunn *et al.*, 1991). The HIV-1 type IIIB gp120-expressing vaccinia virus, vSC-25, kindly provided by Bernard Moss, has been described by Chakrabarti *et al.* (1986) and was used to infect the P815 target cells. For the preparation of high titre virus stocks, CV-1 cells were grown to confluence. At 5 days after infection (m.o.i. of 1) the cells were harvested, resuspended in a small volume of PBS and sonicated three times for 10 s each at 150 W. The cell debris was discarded and the supernatant containing the enriched virus preparation was centrifuged over a 37·5 % sucrose cushion using a Kontron TFT41.14 rotor (36000 r.p.m. for 20 min at 4 °C). The pelleted virus was resuspended in a small volume of PBS and was centrifuged and resuspended once more as above.

Detection and quantification of chimeric proteins. Expression of the chimeric proteins in cells infected with the recombinant VVs was tested by Western blot and immunoprecipitation analysis as previously described (Sambrook *et al.*, 1989). Renaturation of the chimeric proteins following electrotransfer onto a nitrocellulose membrane was achieved by incubation of the Western blots in decreasing urea and DTT concentrations, diluted in PBS starting at 250 mM-DTT and 6 M-urea at 37 °C. Yields of the chimeric proteins were determined from crude cell lysates, after sonification of the infected cells, using a commercial antigen capture assay (Abbott).

Synthetic peptides and oligonucleotides. Peptides were synthesized in a 9050 peptide synthesizer (Milligen) using Fmoc-protected amino acids and were purified by reverse phase HPLC as described previously (Modrow *et al.*, 1989). Oligonucleotides were synthesized in a Milligen DNA synthesizer.

CTL generation. BALB/c mice were immunized intravenously with 10^8 p.f.u. recombinant VV. After 8 weeks, immune spleen cells (5×10^6/ml in 24-well culture plates in complete T cell medium, comprising a 1:1 mixture of RPMI 1640 and EHAA medium containing 10% FCS, 2 mM-L-glutamine, 100 units/ml penicillin, 100 μl/ml streptomycin and 5 μM-2-mercaptoethanol) were restimulated *in vitro* for 6 days with 2·5 × 10^6 P815 cells/ml which had been infected with recombinant VV (for 1 h at 37 °C, 1 p.f.u./cell). After incubation with the virus, target cells were washed three times with PBS before addition to the spleen cells. This procedure was described previously and allows the stimulation of CTL over a limited period without resulting in a detectable lysis of the effector cells (Takahashi *et al.*, 1988, 1989; Buseyne *et al.*, 1993). Alternatively, spleen cells were stimulated *in vitro* with peptide-pulsed syngenic cells (2·5 × 10^5 cells/ml were incubated with the peptides, V3c-16, $V3_{LAI}$ -16 or nef-16, at 1 μM for 4 h, washed three times in PBS and then added to the splenocytes; Takahashi *et al.*, 1992) in 10% rat concanavalin A supernatant-containing medium (rat T cell monoclone; Collaborative Research; Takahashi *et al.*, 1988).

CTL assay. Cytolytic activity of stimulated splenocytes was measured in a 3 h ^{51}Cr-release assay as described previously by Takahashi *et al.* (1988). For testing the peptide specificity of the CTLs, P815 target cells (1×10^6/ml) were incubated for 4 h at 37 °C with the appropriate peptides (1 μM) and labelled for 1 h with 100 μCi/ml $Na_2{}^{51}CrO_4$ (Amersham). All peptides used were shown to be non-toxic for the labelled target cells at concentrations up to 100 μM. Before use, target cells were carefully washed at least three times in RPMI 1640 containing 2% FCS, to remove free peptide and ^{51}Cr.

Alternatively, target cells were infected for 1 h with the described recombinant VVs (10 p.f.u./cell, 37 °C) and were then washed with RPMI containing 1640 2% FCS, to remove free virus. The infected cells were then incubated overnight to allow the expression of the foreign genes from the VV 7·5K early/late promoter and were labelled for 1 h in the presence of ^{51}Cr (100 μCi/ml) as described above. This procedure does not result in virus-mediated lysis of the target cells or even the generation of free virus within the test interval.

Before use, the infected target cells were again washed at least three times in RPMI 1640/2% FCS to remove free ^{51}Cr and were distributed at a concentration of 5×10^3 cells/well in 0·1 ml of medium in round-bottom 96-well plates. Various concentrations of the effector cells in 0·1 ml (in triplicate) were added (50:1 to 1:1). The plates were incubated at 37 °C in 5% CO_2 for 3 h and the cells were then centrifuged at 150 *g* for 5 min. Supernatants were collected and ^{51}Cr c.p.m. was measured in a gamma counter. The percentage specific ^{51}Cr release achieved for the indicated effector to target (E:T) ratios was calculated as 100 × [experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was determined from target cells prepared as described above and incubated without added effector cells. Spontaneous release was below 10% in all experiments. S.E.M.s of triplicate cultures were always less than 4% of the mean.

Results

For the application of artificially designed chimeric proteins to immunization against HIV it is important to know to what extent flanking sequences or local conformational changes at an antigenic site might contribute to antigen processing and consecutive class I MHC-restricted recognition of the antigenic peptide at the cell surface.

To address this question in HIV infections three different p55^{gag}/V3 chimeric genes were established at the DNA level. Three regions within the p55^{gag} precursor, located at the p17-MA/p24-CA cleavage site (amino acids 99 to 154), within the p24-CA domain product (amino acids 211 to 241) and within the p6-LI portion of p55^{gag} (amino acids 436 to 471), were replaced by the coding sequence of the V3 domain and recombined into VV strain Tien Tan (v-TT). The recombinant viruses were v-p55^{gag}/V3c-1, -2 and -3, depending on the location of the V3 domain within the p55^{gag} precursor (Fig. 1). Correct expression of the chimeric proteins in P815 mastocytoma cells was detected by a MAb (16/4/2) which specifically recognizes amino acids 307 to 336 within the p24-CA portion of p55^{gag} (Wolf *et al.*, 1990), demonstrating that the overall amount of protein was comparable in all cell lysates considered (Fig. 2*a*). Using a commercial antigen capture assay, we calculated the amount of recombinant antigen to be 1·0 to 1·2 ng/10^6 infected cells. Shifts in the electrophoretic mobility of the chimeric polypeptides in relation to p55^{gag} chiefly correlated with the number of deleted amino acids. The antigenicity of the inserted V3c domain was proved by a commercial $gp120_{LAI}$-specific murine MAb recognizing a 15-mer V3 peptide (RIQRGPGRAFVTIGK). Detection of the p55^{gag}/V3c chimeric proteins with this LAI isolate-specific MAb confirmed the cross-reactive properties of the inserted V3c sequence (Fig. 2*b*). The different

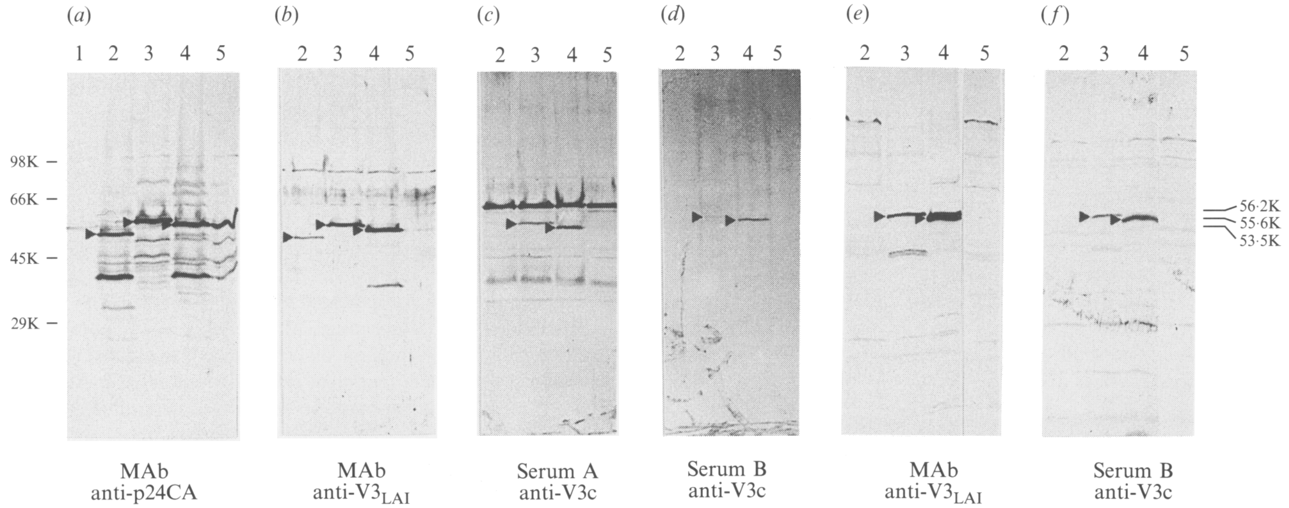

Fig. 2. Analysis of p55V3 chimeric proteins, produced in P815 cells infected with various recombinant vaccinia viruses, by SDS–PAGE followed by immunoblotting. p55V3 chimeric proteins were detected by a MAb to p24 (*a*), by a gp120-V3 loop-specific MAb (*b*) and by two different V3-specific polyclonal antisera raised in rabbits (*c*, *d*). (*e*, *f*) Immunoprecipitation analysis of lysates of P815 cells after infection with VVs. p55V3 chimeric proteins were precipitated (*e*) by the V3 loop-specific MAb and (*f*) by one of the V3-specific polyclonal rabbit antisera. Lane 1, wild-type vaccinia virus; lane 2, v-p55V3c-1; lane 3, v-p55V3c-2; lane 4, v-p55V3c-3; lane 5, v-p55. Shifts in the electrophoretic mobility chiefly correlate with the predicted M_r of the recombinant proteins, indicated on the right. Specifically detected chimeric proteins are labelled by arrows. Positions of the M_r markers are given on the left.

$p55^{gag}$/V3c chimeric proteins were also recognized by polyclonal antisera raised in rabbits towards the V3c peptide coupled to ovalbumin (serum A) or the free V3c 36-mer loop peptide (V3c-36; serum B) (Fig. 2*c*, *d*). These antisera had previously been shown to contain conformation-dependent, in addition to sequence-specific, antibodies (Wagner *et al.*, 1992*b*). According to the position within $p55^{gag}$ recognition of the V3c domain by the V3$_{LAI}$-specific MAb, as well as by the V3c-specific antisera, reproducibly differed among the cell lysates considered (Fig. 2*b* to *d*). The antigenicity of the V3c domain, which was very weak within $p55^{gag}$/V3-1, could be clearly improved after insertion into more carboxy terminal portions of the $p55^{gag}$ carrier protein, as demonstrated for $p55^{gag}$/V3-2 or $p55^{gag}$/V3-3. The protein of M_r 66K recognized in Fig. 3(*c*) is due to a cross-reaction of serum A with albumin. Major changes in the antigenicity might correlate with varying accessibility of the inserted V3c domain for V3-specific antibodies. To confirm this observation under altered, native conditions, we performed an immunoprecipitation analysis of lysates of P815 cells after infection with recombinant VVs using the V3$_{LAI}$-specific MAb and a polyclonal V3-specific antiserum (serum B). The results (depicted in Fig. 2*e* and *f*) show that the position of the V3c domain within the $p55^{gag}$ precursor clearly determines the accessibility of the V3 epitope for specific antibodies. Neither the V3$_{LAI}$-specific MAb nor the V3c-specific polyclonal antiserum precipitated the $p55^{gag}$/V3-1 chimeric antigen, indicating that the V3c domain in this chimera is not accessible to V3-specific antibodies. Confirming the Western blot data, the antigenicity improved following insertion of the V3c domain into positions −2 and −3 (Fig. 2*e* and *f*).

In order to investigate the influence of flanking sequences and changes in local conformations on the priming of V3c-specific CTLs, BALB/c mice were stimulated *in vivo* by recombinant VVs (v-p55V3-1, -2, -3; 10^8 p.f.u./mouse), described above. Syngenic p815 target cells were used for a 7-day-long *in vitro* restimulation of the isolated splenocytes (2 months post-infection) and as targets in a 3 h cytolytic assay. Previous experiments clearly demonstrated that V3-specific killing of target cells strictly correlates with the length and concentration of the V3 peptides used ranging from 10 μM to 10 nM, depending on the E:T ratio used (Takahashi *et al.*, 1988; Wagner *et al.*, 1992*b*). For this purpose, target cells were either incubated with the indicated V3 peptides (1 μM) or were labelled as targets by infection with the recombinant VVs (m.o.i. of 1) as described in Methods. The data summarized in Fig. 3 represent the mean percentage of specific lysis in three replicate cultures.

In initial experiments (Fig. 3*a* to *c*, i) splenocytes of BALB/c mice (H-2^d) were restimulated *in vitro* by syngenic targets infected with the same recombinant VV that was used for the immunization. This procedure has been described by several groups and allows the stimulation of CTLs over a limited period without resulting in detectable lysis of effector cells within the test interval (Takahashi *et al.*, 1988, 1989; Buseyne *et al.*, 1993). Restimulated effector cells were tested for cytolytic

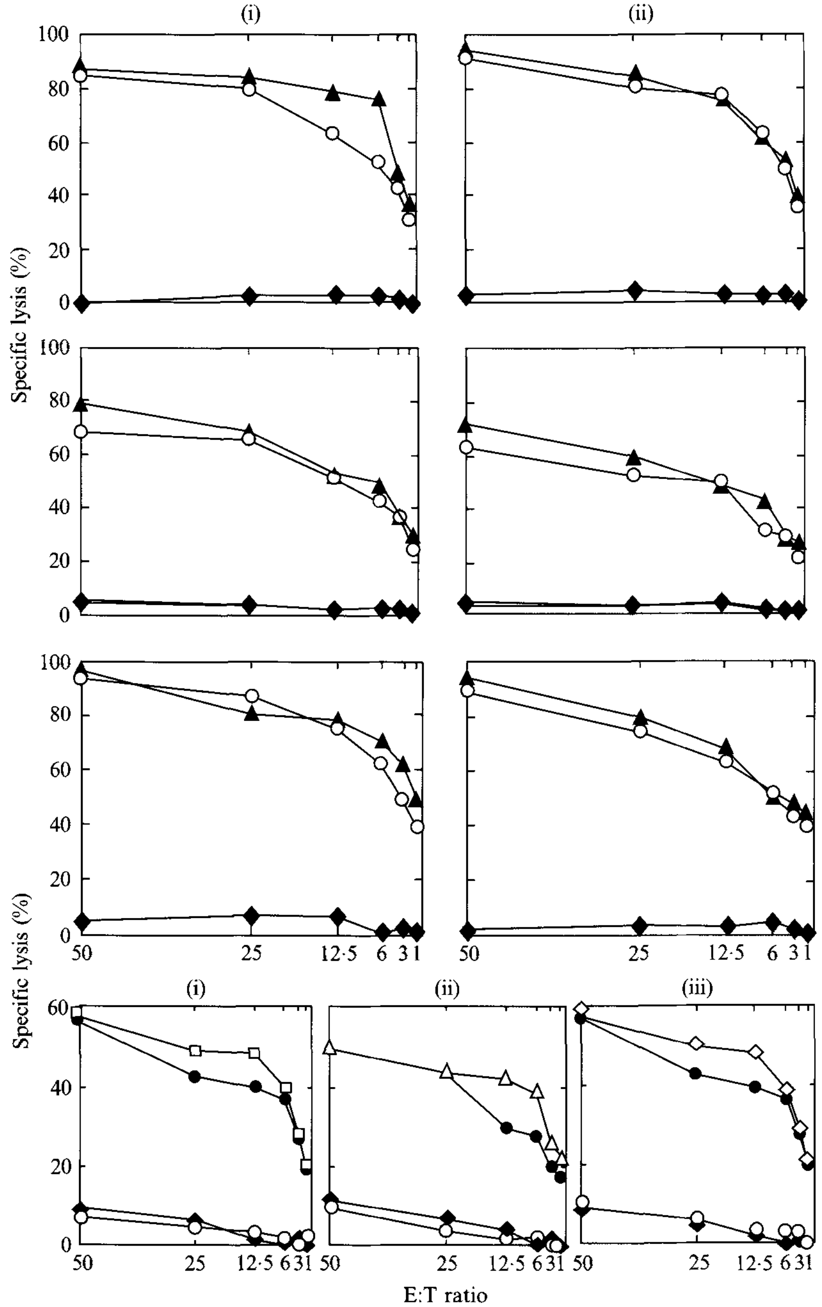

Fig. 3. Induction of $V3_{LAI}$- or treatment with peptide V3c-16, and V3c-specific CTLs after immunization of BALB/c mice with recombinant VV as follows. (*a*) *In vivo* v-p55V3c-1, *in vitro* restimulation with v-p55V3c-1 (i) or V3c-16 (ii). (*b*) *In vivo* v-p55V3c-2, *in vitro* restimulation with v-p55V3c-2 (i) or V3c-16 (ii). (*c*) *In vivo* v-p55V3c-3, *in vitro* restimulation with v-p55V3c-3 (i) or V3c-16 (ii). (*d*) *In vivo* with v-p55V3c-1 (i), v-p55V3c-2 (ii) or v-p55V3c-3 (iii) and *in vitro* with V3c-16 in all cases (see Methods). Peptides in (*a*), (*b*) and (*c*) are marked ◆ nef-16, ▲ V3c-16 and ○ $V3_{LAI}$-16. Recombinant VV in (*d*) are marked □ v-p55V3c-1, △ v-p55V3c-2, ◇ v-p55V3c-3, ● v-gp120, ◆ v-p55gag and ○ v-WT.

activity against P815 cells pulsed with the peptides described in Methods. Only recombinant virus expressing v-p55^{gag}/V3-1, -2 or -3 (Fig. 3*a* to *c*, i), not a control virus expressing the HIV-1 *gag* gene alone (v-p55^{gag}; not shown), could restimulate the effector cells *in vitro* to kill V3 peptide-pulsed P815 targets. Lysis of target cells was observed irrelevant of whether the homologous 16-mer V3c peptide (V3c-16; RIRIGPGRAFVTIGKI) or the heterologous $V3_{LAI}$ peptide ($V3_{LAI}$-16; RIQRGPGRAFVTIGKI) was used for precoating target cells. Pulsing the target cells with the peptide nef-16, LDLWIYHTCGYFPDWQNYTPG, and incubation with the *in vitro* restimulated effector cells did not result in a specific lysis exceeding the spontaneous release, which was below 7% for all peptides tested (1 μM). Splenocytes that were not restimulated *in vitro* did not recognize target cells following incubation with peptide V3c-16, with peptide $V3_{LAI}$-16, or with control peptide nef-16 (not shown). These results demonstrated that the sequence variations between both tested V3 peptides did not affect recognition by the splenocytes. Altered residues flanking the translocated V3c loop as a result of the varying positions of the V3c sequence within $p55^{gag}$ had only a limited influence on the *in vivo* induction of CTLs. Insertion of the V3 domain into the p24-CA moiety of $p55^{gag}$ (v-$p55^{gag}$/V3c-2), however, resulted in a slightly but reproducibly reduced specific lysis (Fig. 3*b*).

Similar results were obtained for each of the tested $p55^{gag}$/V3c chimeric antigens, when peptide V3c-16, not the control peptide nef-16 (not shown), was used for the *in vitro* restimulation. These data confirmed the cross-reactive properties of the V3c-primed CTLs with the LAI strain $V3_{LAI}$-16 peptide (Fig. 3*a* to *c*, ii). In addition, we confirmed that altered flanking of the V3 domain had only a limited influence on the induction of V3-specific CTLs. Target cells pulsed with the peptide nef-16 as described above were not recognized. For BALB/c mice immunized with v-$p55^{gag}$/V3c-2, however, *in vitro* restimulation with V3c-16 reproducibly resulted, as with restimulation with the chimeric VV, in slightly decreased levels of lysis of the target cells. Control experiments using v-$gp120_{LAI}$ for the *in vivo* stimulation of BALB/c mice gave identical results (Wagner *et al.*, 1992*b*).

In order to compare processing and presentation of the V3 antigenic peptide from the described $p55^{gag}$/V3 chimeric proteins to that of the original $gp120_{LAI}$ external glycoprotein, splenocytes of BALB/c mice, immunized with the appropriate chimeric VVs, were stimulated *in vitro* by P815 cells pulsed with peptide V3c-16 or nef-16 (Fig. 3*d*). Target cells were infected with the chimeric $p55^{gag}$/V3 VVs, with v-$gp120_{LAI}$ and, for the control, with v-$p55^{gag}$ and wild-type VV (v-WT). Placing the V3 epitope in different positions in the chimeric proteins in this study did not lead to significant differences in the efficiency of recognition of the V3 epitope by specific CTLs if compared to authentic $gp120_{LAI}$. Target cells infected by v-55^{gag} or by v-WT were not killed (Fig. 3*d*). Splenocytes that were restimulated by control peptide nef-16 did not recognize the pretreated target cells. *In vitro* restimulation of splenocytes with v-WT and infection of the targets by the indicated recombinant VVs resulted, due to the induction of VV-specific CTLs, in specific lysis ranging from 60% to 20% depending on the E:T ratio used in the assay (not shown).

Treatment of the CTL effector cells with anti-Lyt2 MAb plus rabbit complement, but not with anti-L3T4 antibody plus complement or with complement alone, led to a loss of killing activity in all cases tested, confirming previous data (Takahashi *et al.*, 1988). This demonstrates that the effector cells recognizing and killing p55V3-1, -2 or -3 and gp120-expressing, as well as V3 peptide-pulsed target cells, are conventional $Lyt2^+$ $L3T4^-$ ($CD8^+$ $CD4^-$) CTLs (data not shown).

In conclusion, our data suggest that the location of the V3 domain within the $p55^{gag}$ carrier protein does not significantly influence the efficiency of recognition of the processed antigenic V3 peptide (Fig. 3*a* to *d*). The results also demonstrate that a selected domain, V3, known to include a CTL epitope can replace different regions within an antigenic carrier protein ($p55^{gag}$) without significant loss of biological activity.

Discussion

Previous data indicated that the number of original residues flanking an optimal CTL epitope seems to play a crucial role in the presentation of an antigenic peptide. Del Val *et al.* (1991) demonstrated that peptide sequences which are tightly flanking an optimal nonameric antigenic recognition sequence of the murine cytomegalovirus immediate early protein pp89 are directly involved in antigen presentation. Lower amounts of the naturally presented antigenic peptide due to processing from an unfavourable site were shown to be responsible for improper antigen presentation. Our data demonstrated that this restriction can be completely overcome by allowing original flanking of the optimal V3 CTL epitope (Takahashi *et al.*, 1988) by 10 amino acids. We could not find a difference between the processing of the V3 antigenic peptide from the p55 carrier protein, known to be translated on free ribosomes, and processing of the original gp120 envelope protein, synthesized at and transported cotranslationally into the endoplasmic reticulum, thus confirming the cytoplasm as the site of glycoprotein fragmentation (Townsend *et al.*, 1986). This considerably extended initial studies of Hahn *et al.* (1991), reporting that the recognition of an immunodominant influenza virus haemagglutinin site by CTLs is independent of the position of the site in the haemagglutinin translation product.

Minor differences in the *in vivo* induction of V3-specific CTL by v-$p55^{gag}$/V3c-2 in comparison to v-$p55^{gag}$/V3c-1 and v-$p55^{gag}$/V3c-3 might be due to an increased overall degradation rate of the latter two, previously suggested to improve antigen presentation slightly (Townsend *et al.*, 1988). Putative conformational effects on local cleavage efficiency are difficult to prove or refute, but might also be involved in correct processing and antigen presentation. Assuming that the varying accessibility of the V3 domain within different chimeras for V3-specific antibodies reflects differences in the protein conformation, including the V3 loop (Fig. 2*b* to *d*), it might be possible that altered flanking sequences influence processing and presentation of a translocated domain from chimeric proteins. These findings and considerations have important implications for the understanding of principles that govern antigen presentation and open new perspectives for a rational vaccine design.

The development of severe immune dysfunctions following HIV infection is supposed to be induced, or at least supported, by the HIV-1 external glycoproteins. Vaccines restricted to immunologically defined epitopes might therefore avoid adverse side-effects. This paper showed that chimeric antigens, expressed via recombinant VVs, are capable of inducing CTLs directed towards a translocated epitope. The insertion of more than one epitope into p55^{gag} by combining different chimeric genes might prove especially useful for those antigenic sites which can be presented by a number of different HLA haplotypes as shown e.g. for epitopes within the reverse transcriptase and the Nef regulatory protein (Walker *et al.*, 1987, 1989; König *et al.*, 1990).

Another important requirement for a rationally designed vaccine should be the induction of a humoral immune response allowing the neutralization of free virus in addition to the CTL-mediated elimination of infected cells. The efficacy of the V3 domain, proved in a series of experiments to be the principal HIV-1 neutralizing determinant (Palker *et al.*, 1988; Goudsmit *et al.*, 1988) has been discussed extensively by Berman *et al.* (1990), Emini *et al.* (1990), Girard *et al.* (1991) and Devash *et al.* (1990). We found this domain was suitable for analysis of the stimulation of both arms of the immune response. Using the p55^{gag}/V3 chimeric VVs as a live vaccine in rabbits, however, only low titres of V3-specific antibodies could be induced. With respect to the generation of a p55-specific humoral immune response, this was expected from previous studies underlining the low immunogenicity of recombinant VVs expressing cytoplasmic proteins (v-p55) in comparison to purified antigen (Wagner *et al.*, 1992*a*). Efficient stimulation of B lymphocytes might require larger amounts of free antigen than that released from cells infected by recombinant VVs. Induction of high titre neutralizing activity, as proved for branched synthetic V3 peptides (Shirai *et al.*, 1992), could present a useful approach in boosting V3-specific antibodies after primary immunization with chimeric VVs described above.

Owing to the intrinsic adjuvant properties and high immunogenicity of particulate structures, alternative carrier systems have been developed for the presentation of heterologous epitopes recently, mainly based on the hepatitis B virus surface antigen, its core antigen or on yeast TyA particles (Kingsman *et al.*, 1989; Beesley *et al.*, 1990; Schlienger *et al.*, 1992). Extensive studies of the p55^{gag} particle-forming capacity (Wagner *et al.*, 1992*a*) and of the identification of domains necessary for correct assembly of the Gag precursor into immature virus-like particles (von Poblotzki *et al.*, 1993) allowed us to establish a novel antigen presentation system. This system is based on recombinant p55^{gag} virus-like particles which were previously shown to be highly immunogenic in rabbits (Wagner *et al.*, 1992*a*). Placing immunologically defined regions such as the V3 loop sequence in different positions of the p55^{gag} precursor leads in some cases to the generation of chimeric p55^{gag}/V3 virus-like particles (R. Wagner *et al.*, unpublished). Further analysis of such virus-like particles will tell us more about the immunogenic potential of chimeric proteins and about the possibility of constructing a safe and effective candidate vaccine.

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