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Presentation of human immunodeficiency virus epitopes by chimeric core particles of hepatitis B virus

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Abbreviations: HBV, hepatitis B virus; HBcAg, hepatitis B virus core antigen; HIV, human immunodeficiency virus; AcNPV, *Autographa californica* nuclear polyhedrosis virus

Abstract

We have constructed a synthetic open reading frame based on amino acid sequences from two conserved regions of the HIV-1 gp120 *env* protein, and from a predicted antigenic region of the p17 *gag* protein. We have synthesized these genes by using the preferred codon usage for the polyhedrin gene of the baculovirus AcNPV. This synthetic gene was fused either at the 5'- or at the 3'-terminus of the human hepatitis B core antigen (HBcAg) gene, and the fused genes were expressed in *Spodoptera frugiperda* cells using recombinant baculovirus. Both fused genes were capable of directing the synthesis of chimeric 27-nm spherical particles. Electron microscopic analyses revealed morphologies distinct from that of HBcAg particles. Antisera directed against either chimeric particles could immunoprecipitate the non-glycosylated form of HIV-1 gp120. In contrast, antisera against only the N-terminal fusion, but not the C-terminal fusion particles, recognized HIV-1 p17. Furthermore, antisera raised against non-glycosylated gp120 immunoprecipitated both types of chimeric particles. Our results demonstrate that the HIV-1 epitopes on the chimeric HBcAg-HIV particles are properly recognized as authentic HIV antigen.

Keywords: HBV, HIV, chimeric protein, epitopes, vaccine

Introduction

The human hepatitis B virus (HBV) core antigen (HBcAg) gene encodes the 183 amino acid, 21-kDa nucleocapsid protein. HBcAg has been expressed in both prokaryotic and eukaryotic heterologous expression systems (Stahl *et al.*, 1982; Will *et al.*, 1984; Miyanohara *et al.*, 1986; Ou *et al.*, 1986; Roossinck *et al.*, 1986; Jean-Jean *et al.*, 1987; McLachlan *et al.*, 1987) including insect cells (Lanford *et al.*, 1988; Takehara *et al.*, 1988). In all systems, HBcAg directs the self-assembly of a 27-nm particle structure which is morphologically and antigenically similar to that of native HBV nucleocapsids isolated from infected human hepatocytes (Cohen and Richmond, 1982).

Despite the fact that it is internal in the virion, HBcAg is highly immunogenic during HBV infection. Cell-mediated immune (CMI) response to HBcAg is thought to be important for the clearance of viral infection (Mondelli *et al.*, 1982; Eddleston *et al.*, 1983). Immunization of chimpanzees with core antigen provides substantial protection against infection with HBV (Murray *et al.*, 1984; Iwarson, *et al.*, 1985; Murray, *et al.*, 1987). This enhanced immunogenicity may be explained by the presence of multiple T helper cell (T_h) epitopes, and by the ability of HBcAg to function as both a T cell-dependent and T cell-independent antigen (Milich and McLachlan, 1986; Milich *et al.*, 1987).

Because of these structural and immunological features, it has been suggested that HBcAg would function efficiently as a carrier protein for heterologous peptide epitopes (Clarke *et al.*, 1987; Milich *et al.*, 1987). Core antigen may enhance the immunogenicity of polypeptides which are linked in a common structure (Milich *et al.*, 1987). In support of this, a number of genetic fusions with HBcAg have resulted in chimeric polypeptides which elicited a good response to foreign epitopes (Clarke *et al.*, 1987; Borisova *et al.*, 1989; Stahl and Murray, 1989; Schodel *et al.*, 1990; Clarke *et al.*, 1990; Moriarty *et al.*, 1990; Schodel *et al.*, 1992).

Good candiates for such a fusion are the human immunodeficiency virus (HIV) envelope glycoprotein (*env*; gp120 and gp41) and nucleocapsid protein (*gag*; p17 and p24) genes. Although high sequence variation is seen in the envelope glycoprotein genes among different HIV isolates, several conserved domains have been a good target for immunotherapy if they are incapable of escape from immune surveillance due to the necessity of structural conservation. Antibodies to *gag* p17 are not protective (Emini *et al.*, 1990), but p17

may be important in stimulating the immune response to HIV (Sarin *et al.*, 1986; Pepsidero *et al.*, 1989). A combination of antigenic epitopes might therefore be more effective in providing protection from disease. Here we report the successful synthesis and isolation of chimeric human hepatitis B virus core particles which carry a series of linked peptides derived from the HIV-1 glycoprotein gp120 and nucleocapsid protein p17.

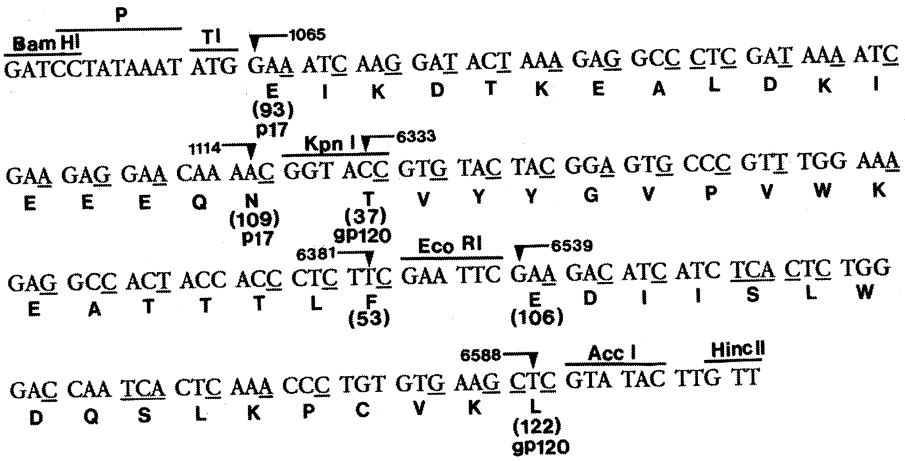

Figure 1. Construction of synthetic HIV-1 DNA sequence. Three pairs of oligonucleotides representing a portion of p17 and two parts of gp120 proteins of HIV-1 were synthesized on the basis of amino acid sequence information from the *gag* and *env* proteins. Codons were altered according to the most frequently utilized codons for the polyhedrin protein of AcNPV. The three pairs of oligounucleotides were ligated together at the *Kpn*I site and the *Eco*RI site. The terminal *Bam*HI and *Hinc*II sites allowed subcloning of the fragment into the multiple cloning site of pUC18. One letter amino acid codes are shown beneath the DNA sequence. The bracketed numbers give the corresponding amino acid position in HIV-1 p17 or gp120. The numbers above the nucleotides give the corresponding nucleotide position in the genome of HXB2 stain of HIV-1 (from *Human Retroviruses and AIDS*, Los Alamos National Laboratory). The nucleotides underlined were substituted for the original nucleotides so as to utilize those codons most frequently used by AcNPV polyhedrin protein. The sequence beneath P is the polyhedrin mRNA leader sequence immediately 5' to the initiation codon.

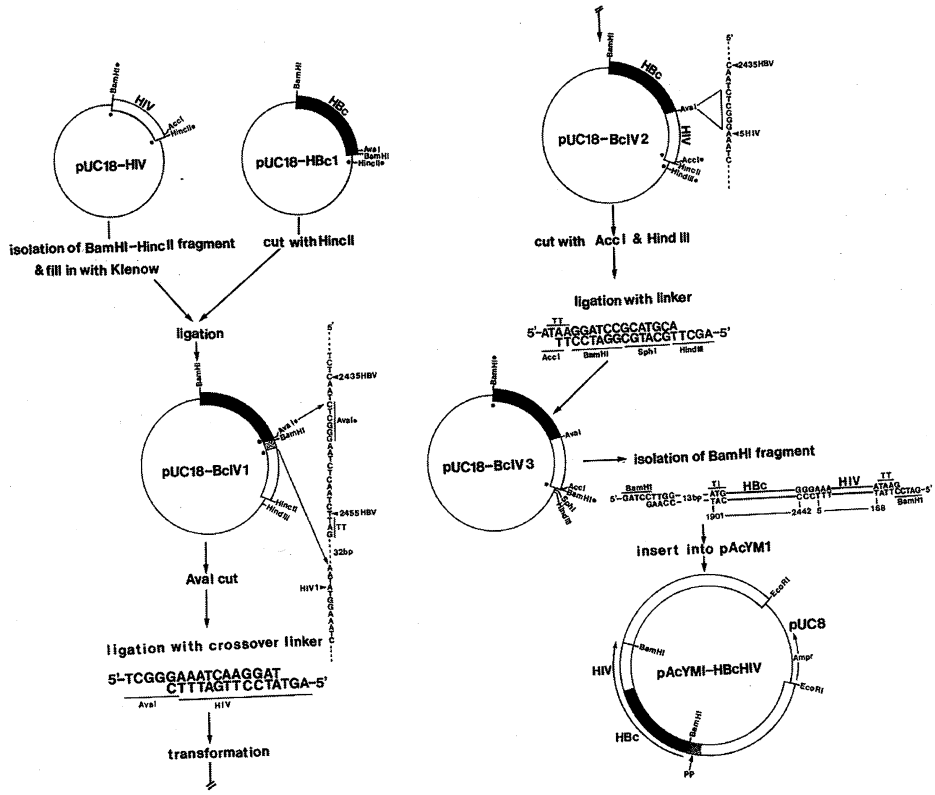

Figure 2. Construction of the transfer vector pAcYM1H.BcHIV. The HIV-1 coding segment from pUC18-HIV was inserted at the *Hinc*II site of pUC18-HBc1 (which has *Ava*I site removed) to create pUC18-BclV1. The sequence between the *Ava*I site of HBcAg and the fourth nucleotide of the HIV-1 coding segment was deleted by cross-over linker directed mutagenesis. A second linker was used to introduce a termination codon followed by a *Bam*HI site. The HBcAg-HIV fusion *Bam*HI fragment was inserted in the correct orientation into pAcYM1. The positions marked 2435 HBV and 2455 HBV are numbered according to the HBV genome as published (Ono *et al.*, 1983). PP indicates the sequence in pAcYM1-HBcHIV which corresponds to the sequence immediately upstream of the AcNPV polyhedrin gene initiation codon.

These particles have been investigated for their structural and antigenic properties.

Methods

Cells and media

Escherichia Coli (*E. Coli*) strain JM101 was used in routine transformations and preparation of DNA. Strain RR1 was used specifically for transformation and propagation of pAcYM1 and its derivatives. SF9, a clonal isolate of *Spodoptera frugiperda* (SF9) cells, was from Dr. M. Summers, Texas A&M University, U.S.A. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and AcNPV-YM1KTc were from Dr. D. H. L. Bishop, NERC Institute of Virology, U.K. *E. coli* strains were grown in LB media. SF9 cells were grown as monolayer cultures in TNM-FH medium containing 10% fetal bovine serum.

Plasmids

Plasmids pAcYM1 and its derivative pAcYM1-KTc, with the HBcAg gene inserted in the unique *Bam*HI site, were from Dr. D. H. L. Bishop. Plasmid pUC18-HBc was a derivative of pUC18 with the *Bam*HI HBcAg insert from pAcYM1-KTc. pUC18-*Ava*I had the *Ava*I site of pUC18 destroyed by Klenow repair. pUC18-HBc1 had the *Bam*HI fragment from pUC18-HBc subcloned into pUC18*Ava*I. pUC18-*Sma*I had the *Sma*I of pUC18 destroyed by Klenow repair.

Oligonucleotides and antisera

Deoxyoligonucleotides were synthesized on an Applied Biosystems 380B DNA systhesizer. Rabbit anti-HBcAg serum was from Accurate Chemical and Scientific Corp., Westbury, N.Y., U.S.A.

Construction of recombinant baculovirus transfer vectors

Three pairs of complementary oligonucleotides (Figure 1) were first annealed, ligated at their compatible *Kpn*I and *Eco*RI termini, and inserted into pUC18 between the *Bam*HI and *Hinc*II sites. The final product, pUC18-HIV (Figure 2), contained a modified HIV-1 coding segment. For construction of a C-terminal fusion with HBcAg (Figure 2), the HIV-1 coding segment was excised and blunt-end subcloned at the *Hinc*II site of pUC18-HBc1. Nucleotides between HBcAg and the HIV-1 segment, and the last 17 base pairs of HBcAg, were removed by cross-over linker directed mutagenesis (Sung *et al.*, 1986; Garson *et al.*, 1990). This plasmid was then linearized and ligated to an oligonucleotide to create a translation termination codon. The resultant *Bam*HI fragment was transferred to the baculovirus transfer vector pAcYM1.

For construction of an N-terminal fusion (Figure 3), the HIV-1 segment from pUC18-HIV was excised and blunt-end subcloned into the *Sma*I site of pUC18-*Sma*I

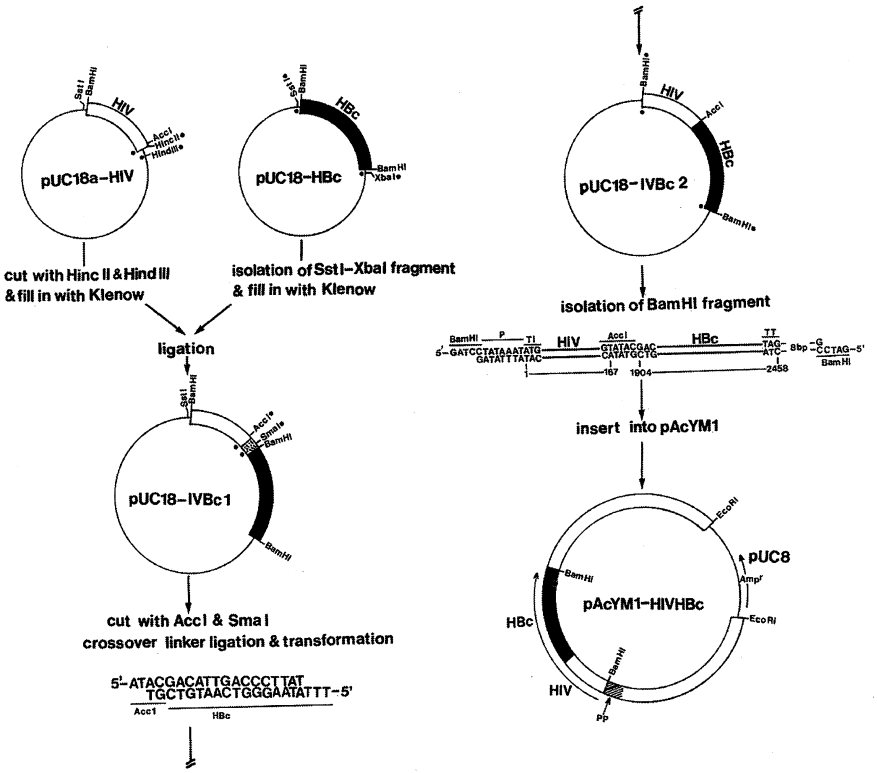

Figure 3. Construction of the transfer vector pAcYM1-HIVHBc. The HBV coding segment of pUC18-HIV was subcloned into pUC18-*Sma*I (with the *Sma*I removed) to form pUC18a-HIV. An HBcAg fragment from pUC18-HBc was blunt-end subcloned in pUC18a-HIV to form pUC18-IVBc1. The sequence between the *Acc*I site of HIV and the fourth nucleotide of the HBcAg open reading frame were deleted by cross-over linker directed mutagenesis. The HIV-1-HBcAg *Bam*HI fusion fragment was inserted in the correct orientation into pAcYM1. Other labels are as indicated in Figure 2.

to create pUC18a-HIV. An HBcAg insert from pUC18-HBc was blunt-end subcloned into pUC18a-HIV. Intervening nucleotides were again removed by cross-over linker directed mutagenesis. The resultant *Bam*HI fragment was transferred to the transfer vector pAcYM1.

In both cases, the final inserts were sequenced to confirm that fusion via cross-over linker directed mutagenesis was in frame.

Isolation of recombinant virus

Techniques for the isolation and manipulation of recombinant baculovirus were performed essentially as previously descfibed (Summer and Smith, 1987; Kang *et al.*, 1988). Culture supernatants were harvested and titrated on SF9 cell monolayers. Recombinant viruses were identified by plaque hybridization essentially as previously described (Summers and Smith, 1987), except that viral plaques were blotted from the agarose overlays removed from infected monolayers. Overlays were stored covered at 4°C during hybridization of the filters. Plaques were picked by aligning the exposed autoradiographic image from the hybridized filter with the agarose overlay. Selected recombinants were purified by three consecutive cycles of plaque isolation.

Expression analysis and immunoblotting

SF9 cells in monolyers were infected with either wild type or recombinant AcNPV at a multiplicity of infection (m.o.i.) of 5 pfu/cell. Cells were harvested 4 days post-infection (p.i.) and resuspended in dissociation buffer (10% β-mercaptoethanol, 10% SDS, 25% glycerol, 10 mM Tris-HCl, pH 6.9, 0.02% bromophenol blue). Samples were analyzed by 12% SDS-PAGE (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose and the Bio-Rad Immuno-Blot AK system was used to detect antibody- antigen reactivity.

Purification of HBcAg and chimeric particles

Cells infected with recombinant virus were harvested 4 days p.i. and resuspended at 4 x 10^7 cells/ml in TNE (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA) or in TNM-FH medium. Cells were lysed by sonication, diluted 1-fold with TNE, and centrifuged at 5,000 *g* for 10 min. Supernatants were collected and centrifuged further at 11,000 *g* for 30 min. Aliquots from this supernatant (0.2 ml) were centrifuged through a 25% sucrose cushion at 87,000 *g* for 5 h. The resultant pellets were resuspended in TNE, sonicated, and centrifuged through a continuous 20-75% renografin-76 (Squibb Inc.) gradient containing 0.1% Triton X-100, at 80,000 *g* for 16 h. Bands were collected, diluted 10-fold in TNE, and material collected by centrifugation at 87,000 *g* for 2.5 h. Pellets were resuspended in TNE and stored at 4°C

Electron microscopy

Samples were negatively stained with 1% uranyl acetate by standard procedure and examined by a Philips EM 301.

Serology and immunoprecipitation

Rabbits were immunized by intramuscular inoculation of purified particles (100 μg per dose) at 2 week intervals. Samples for first injections were prepared in Freund's complete adjuvant, and samples for subsequent injections were prepared in incomplete adjuvant. Serum samples were collected before the first injection and 10 days after each subsequent injection. For immunoprecipitation, SF9 cells infected in monolayer with recombinant baculovirus were starved at 2 days p.i. for 1 h with methionine-free Grace's medium supplemented with ^{35}S-methionine (at ~100 μCi/ml) for 1 h. Cells were washed with cold PBS once and harvested by scraping. Labelled cells were lysed with detergent (1% NP40, 150 mM NaCl, 10 mM EDTA) on ice for 20 min. Lysates were centrifuged in a microfuge to clear cellular debris, diluted 5-fold with RIPA buffer (1% NP40, 1% Sarcosyl, 0.1% SDS 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 6.9), and incubated with the appropriate antisera at a dilution of 1:100 at 4°C overnight. Antigen-antibody complexes were recovered by incubating with 5 mg/ml protein A-Sephadex beads at 4°C for 1 h. The beads were centrifuged briefly in a microfuge to recover and washed twice with RIPA buffer and once with 0.1% NP40 in 10 mM Tris-HCl (pH 7.5). Immunoprecipitated protein was recovered by boiling the beads in sample buffer, and aliquots of the supernatant were analyzed by 12% SDS-PAGE. Gels were soaked in Amplify (Amersham), dried, and exposed to Cronex X-ray film (Dupont).

Result

Expression of HBcAg-HIV-1 fusion proteins in SF9 cells

We have synthesized three sets of oligonucleotides pairs designed to be joined in-frame (Figure 1). The first pair encodes 17 amino acids representing a portion of the HIV-1 p17 *gag* protein which is predicted to be immunogenic based on its relative hydrophilicity (residues Glu at 93 to Asn at 109). The remaining two pairs encode peptides representing conserved domains from HIV-1 gp120 (residues Thr at 37 to Phe at 53, and Glu at 106 to Leu at 122). When these segments are linked together and fused in-frame to the 3' terminus of the HBcAg gene, they encode 55 amino acids including four extraneous amino acids created by restriction endonuclease site linkages, and the last three amino acids of the core protein are deleted (Figure 2). When

the synthetic HIV-1 coding segment is fused to the 5' terminus of the HBcAg gene, it encodes 57 amino acids including five extraneous amino acids created by restriction endonuclease site linkages, and replaces the N-terminal methionine of the native core protein (Figure 3). We have used the technique of cross-over linker directed mutagenesis to remove non-coding flanking nucleotide sequences and to place these fusions in-frame (Figure 2 and 3). The nine nucleotide sequence CCTATAAAT from the 5' non-coding region of the AcNPV polyhedrin protein was included in the chimeric oligonucleotide. This sequence is thought to be important to the high-level expression of the polyhedrin protein (Matsuura et al., 1987; Luckow and Summers, 1988), and its placement next to the start codon of the HIV-1 coding segment should allow efficient translational initiation when fused to the N-terminus of HBcAg.

After fusion of the HIV-1 coding segment either at the 5' or at the 3' ends of HBcAg, the two completed constructs were inserted into the AcNPV transfer vector pAcYM1 and the corresponding recombinant baculoviruses, AcHBcHIV and AcHIVHBc, were isolated (Figure 2 and 3). These viruses were used to infect SF9 cells, and total infected cell protein was analyzed by SDS-PAGE. Recombinant baculovirus, AcYM1KTc, was used to produce HBcAg without fusion. Both recombinant viruses, AcHBcHIV and AcHIVHBc, produced large quantities of recombinant chimeric protein (HBcHIV and HIVHBc, respectively; Figure 4a). The molecular weights of the chimeric proteins were approximately 29,000 and 29,500 respectively, which corresponds with the predicted sizes. In other experiments these proteins appeared to be somewhat larger (e.g. Figure 7). These proteins were not seen in uninfected cells or in wild type AcNPV infected cells (Figure 4a, lanes C and W). The level of expression of both proteins is similar to that seen for HBcAg alone (Figure 4, compare lane 1 with lanes 2 and 3). Protein samples were blotted and analyzed with rabbit anti-HBcAg serum (Fig. 4b). HBcAg and both chimeric proteins were reactive with anti-HBcAg serum, confirming the presence of the HBcAg antigenic site in the chimeric proteins.

Purification of HBcAg-HIV-1 chimeric particles

We examined whether the chimeric HBcAg-HIV constructs were capable of assembling into 27-nm HBcAg particle-like structures. SF9 cells were infected with the recombinant viruses AcYM1KTc, AcHBcHIV and AcHIVHBc, and cytoplasmic extracts were prepared and analyzed by SDS-PAGE. Approximately 50-70% of the total chimeric proteins were recovered in this fraction (data not shown). The HBcAg chimeric

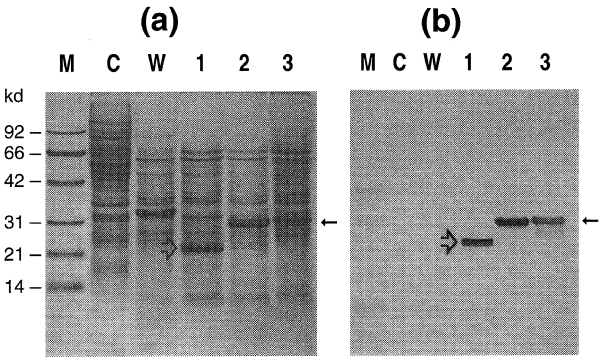

Figure 4.
(a) SDS-polyacrylamide gel electrophoresis of proteins from recombinant AcNPV infected cells. Infected or uninfected cells were lysed by boiling in SDS sample buffer and loaded at approximately 8 μg per well. The gel was stained with Coomassie Blue. M: molecular weight standard; C: uninfected cell control; W: wild type AcNPV infected cell; lane 1: AcNPVKTc infected cell; lane 2: AcNPV-HBcHIV infected cell; lane 3: AcNPV-IVHBc infected cell. The open arrow shows the position of expressed HBcAg, the closed arrow shows the position of expressed fusion protein.
(b) Western blot analysis. Anti-HBcAg serum was diluted 1:3,000 and incubated with the blotted membrane for 1 h before the addition of second antibody (1:3,000). Samples are ordered and labelled as in (a).

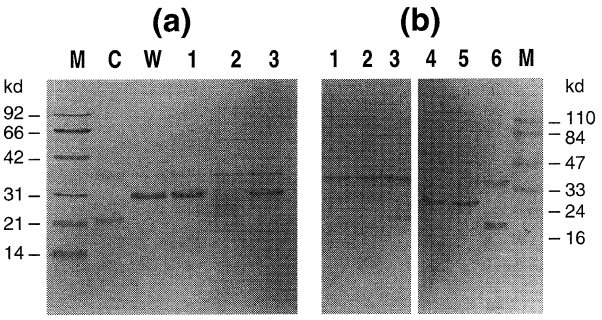

Figure 5.
(a) SDS-Polyacrylamide gel electrophoresis of purified HBcAg and chimeric particles. Purified protein was boiled in sample buffer and loaded approximately 2 μg per well. The gel was stained with Coomassie Blue. M: Molecular weight standard; lane 1: HBcAg particles; lane 2: HBcHIV particles; lane 3: HIVHBc particles from cells resuspended in TNM-FH after harvest and prior to sonication; lane 4: HIVHBc particles from cells resuspended in TNE buffer after harvest; lane 5: as in lane 4, but cells were immediately heated at 65°C after resuspension.
(b) Western blot analysis. Antisera were diluted 1:300 and incubated with the blotted membranes for 1 h before the addition of second antibody (1:3,000). M: prestained molecular weight marker; lanes 1 and 4: HIVHBc; lanes 2 and 5: HBcHIV; lanes 3 and 6: HBcAg. Lanes 1-3 were incubated with anti-BcHIV serum, lanes 4-6 were incubated with anti-HIVHBc serum.

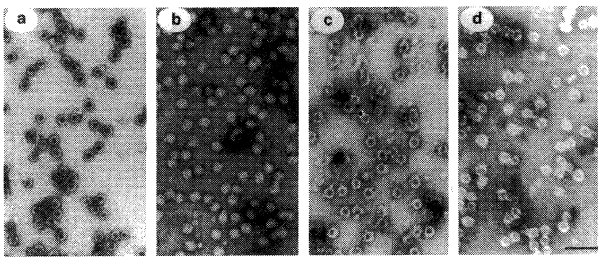

Figure 6. Electron micrographs of purified particles from preparations of JBcAg and its derivatives. HBcAg particles **(a)**, HBcHIV chimeric particles **(b)** and HIVHBc **(c)** chimeric particles from cells resuspended in TNM-FH after harvest and heat treated at 65°C. **(d)** HIVHBc chimeric particles from cells resuspended in TNE after harvest.

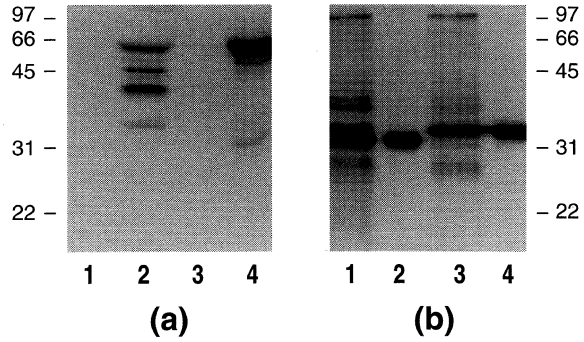

Figure 7. Immunoprecipitation of nonglycosylated gp120 (p53) and HBcAg-HIV-1 chimeric particles. **(a)** Cells infected with Acgp120-Ds were labelled with ^{35}S-Met at day 2 p.i. before lysates were incubated. Lane 1: preimmune rabbit sera; lane 2: anti-HBcHIV sera; lane 3: anti-HIVHBc sera; lane 4: anti-p53 sera. Immunoprecipitated protein was recovered with protein-A Sephadex and dissociated by boiling in sample buffer before SDS-PAGE. **(b)** Either total labelled protein (lanes 1 and 3) or protein immunoprecipitated with anti-p53 sera (lanes 2 and 4) were loaded from cells infected with either AcHBcHIV (lanes 1 and 2) or AcHIVHBc (lanes 2 and 4).

particles were purified by centrifugation through a 20-75% renograffin-76 density gradient. Several bands were visually observed on these gradients, some of which were likely composed of baculovirus components. The position of the major band from the AcYM1KTc infected sample was used as a guide to select a corresponding band from the AcHBcHIV and AcHIVHBc infected samples. The composition of the recovered bands was determined by SDS-PAGE analysis. For AcYM1KTc, the band was primarily composed of 22-kDa HBcAg, plus a 36-kDa minor protein (Figure 5A, lane 1). Likewise, the AcHBcHIV band contained the 29-kDa fusion protein along with the 36-kDa species (Figure 5A, lane 2). In contrast, the AcHIVHBc band also contained a number of major species smaller than that predicted for HIVHBc fusion protein. These species could be completely eliminated if infected cells were resuspended in TMN-FH medium rather than hypotonic TNE buffer prior to lysis by sonication. They could also be eliminated if infected cells were heated at 65°C for 15 min in TNE prior to sonication (lane 5). These results suggest that HIVHBc is especially susceptible to a heat-sensitive protease specie(s) in SF9 cells

Electron microscopy of purified HBcAg, HBcHIV and HIVHBc particles

The morphologies of gradient purified HBcAg, HBcHIV and HIVHBc particles were assessed by electron microscopy. HBcAg was evident as small spherical particles of approximately 27 nm, as expected. These particles were penetrated by stain, demonstrating a regular interior cavity (Figure 6a). AcHBcHIV also produced spherical particles which appeared to be slightly larger than the HBcAg particles (Figure 6b). However, the HBcHIV particles had an opaque centre not penetrated by the stain. In contrast, HIVHBc particles had a penetrable cavity similar to that of HBcAg particles (Figure 6c and 6d). In addition, HIVHBc particles which were heat-treated had a more indistinct surface outline, with the presence of irregular surface ruffles (Figure 6c). Particles which were allowed to partially degrade (not heat-treated) retained the indistinct outline, but many of them were devoid of surface ruffles (Figure 6d).

These data strongly suggest that when fused to the C-terminus of HBcAg, the HIV-1 peptide is disposed interior to the 27 nm particle, creating a densely packed interior cavity. In contrast, when the HIV-1 peptide is at the N-terminus, it appears to be disposed exterior to the particle, creating surface ruffles. The peptide also appears to be susceptible to proteolytic cleavage when fused at the N-terminus, characterized by a decrease in molecular weight for the polypeptide and a loss of surface ruffles from the chimeric particle.

Immunogenicity of the chimeric praticles

In order to test whether the chimeric HBcAg-HIV-1 particles were immunogenically similar to the native virion proteins, purified chimeric particles were used to raise antisera. By immunoblot analysis, antisera were shown to be reactive to the original chimeric particles used for immunization. Antisera to HIVHBc (Figure 5b, lanes 1-3) and HBcHIV (Figure 5b, lanes 4-6) were reactive with both HBcAg and the HBcAg-HIV fusions. The antisera also recognized the 36-kDa protein. These results demonstrate that the addition of the foreign epitopes has not significantly changed the antigenicity of HBcAg. In the case of the rabbit anti-HIVHBc serum, reactivity to the 36-kDa protein appears

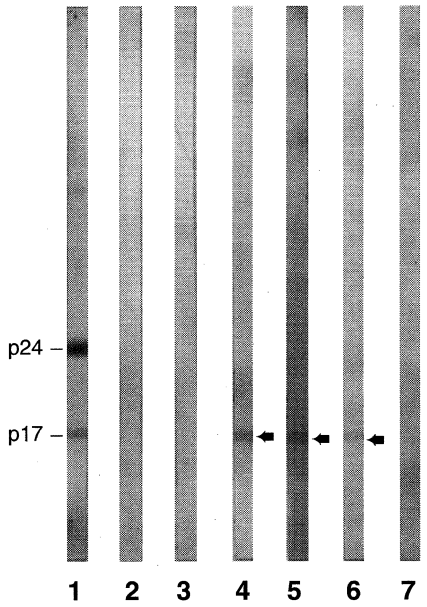

Figure 8. Western blot analysis of HIV virion protein using anti-chimeric particle sera. Biotech/Dupont HIV Weatern blot strips were incubated with rabbit anti-HIV-2 gag polyprotein serum (lane 1); with anti-HBcAg serum (lane 2); preimmune serum (lane 3); with anti-HIVHBc serum from 2nd inoculation (lane 4); with anti-HIV serum from 3rd inoculation (lane 5); with anti-HBcHIV serum from second inoculation (lane 6); with anti-HBcHIV serum from 3rd inoculation (lane 7). Strips were incubated with sera diluted 1:200 for 4 hr before addition of second antibody (1:3,000).

to be favoured over the recombinant proteins, while the opposite appears to be true for the rabbit anti-HIVHBc serum. Both antisera react equally well to the two chimeric proteins.

Antisera against the chimeric particles were tested for their ability to immunoprecipitate the non-glycosylated form of HIV-1 gp120 (53-kDa protein), expressed in SF9 cells by infection with a recombinant baculovirus (Li *et al.*, 1993). This non-glycosylated gp120 polypeptide displays reactivity to AIDS patient sera not significantly different from secretory, glycosylated gp120 (Li *et al.*, 1993). It therefore represents an appropriate target to test reactivity of the chimeric particle antisera against HIV-1 gp120. Antisera against both HBcHIV and HIVHBc particles immunoprecipitated the 53-kDa protein and some degraded forms of gp120 from lysates of infected cells (Figure 7a, lanes 2 and 3). Anti-HIVHBc serum precipitated the 53 kDa protein weakly. In this particular experiment, the antisera used was collected after the first inoculation of HIVHBc, and showed a low level of reactivity to the 53 kDa protein. In a reciprocal experiment, antisera raised in rabbits against the 53-kDa protein immunoprecipitated both HBcHIV and HIVHBc (Figure 7b, lanes 2 and 4).

These results demonstrate an antigenic similarity in structure between the HIV-1 oligopeptide within chimeric particles and non-glycosylated form of HIV-1 gp120. In contrast, we were unable to immunoprecipitate HIV-1 *gag* p17 from cells infected with a recombinant baculovirus (data not shown). This suggests that this portion of the oligopeptide may differ significantly in structure from native p17.

We tested the reactivities of our fusion particle antisera against Biotech/Dupont Western Blot strips prepared from HIV-1 virions. Figure 8. shows the Biotech/DuPont strips tested with antisera against HBcAg particles (lane 3), HIVHBc particles (lanes 4 and 5), and HBcHIV particles (lanes 6 and 7). We found that the anti-HIVHBc sera reacted with *gag* p17 (lanes 4 and 5), while anti-HBcAg and anti-HBcHIV sera did not. Antisera against the *gag* polyprotein of HIV-2 has been demonstrated to be crossreactive to p24 and p17 of HIV-1 gag (Luo *et al.*, 1990). We have used this serum to identify the HIV-1 *gag* proteins (Figure 8, lane 1). Control rabbit serum and antiserum against HBcAg were not reactive with any HIV1 proteins (lanes 2 and 3, respectively). No reactivity to gp120 was seen with antisera directed against chimeric particles. Furthermore, no cross-reactivity between the 53-kDa protein and the chimeric particles could be seen by Western Blot analysis (data not shown). This suggests that antisera to the fusion particles is only reactive to linear epitopes of non-denatured p53.

Discussion

We have expressed two chimeric proteins using the HBV core antigen fused to an HIV-1 oligopeptide. This oligopeptide contains three different segments (one from p17 and two from gp120) and was fused either at the N-terminus or at the C-terminus of HBcAg. This represents a more recent approach to synthetic vaccine construction in which heterologous peptide epitopes are fused to a common protein carrier. HBcAg appears to be ideal in this regard. It can self-direct the assembly of a 27-nm particle structure in the absence of any other viral products. This particulate structure should be advantageous in that it would allow presentation to the immune system of a high copy number of foreign epitopes on a structure similar to that of a native viral nucleocapsid. HBcAg is also highly immunogenic. It has multiple T-cell epitopes, and can act as both a T-cell dependent and a T-cell independent antigen (Milich and McLachlan, 1986; Milich *et al.*, 1987).

This use for HBcAg was proven feasible in a study in which 19 amino acids of the foot and mouth disease virus VP1 protein were fused N-terminal to the core protein (Clarke *et al.*, 1987). The fused oligopeptide was dramatically more immunogenic than the peptide

administered alone. Subsequently, the formation of chimeric 27-nm particles involving several different heterologous peptides has been demonstrated. These particles have uniformly been immunogenic for the heterologous peptides. HBcAg appears to be superior to the HBV surface antigen as a generic carrier system, as it doesn't appear to be conformationally sensitive to foreign peptide addition (Bruss and Ganem, 1991).

We have been able to easily purify large quantities of chimeric 27-nm particles from recombinant baculovirus infected SF9 cells. The purity of these particles was sufficiently high to allow preliminary testing, and could easily be improved by further separation. While a considerable proportion of these recombinant polypeptides appeared to be membrane-bound and pelleted with cell debris, they did not differ in this from native core protein. Thus the addition of foreign epitopes to the core protein did not appear to cause any gross conformational alterations affecting intracellular solubility. Furthermore, the chimeric particles from infected SF9 cells were hydrodynamically similar to HBcAg 27-nm particles based on their similar position in a renografin-76 buoyant density gradient.

These chimeric particles were morphologically distinct from native 27-nm particles. An exterior disposition of the HIV-1 oligopeptide in HIVHBc particles was suggested by the irregular surface ruffles and indistinct outline, and the susceptibility of this protein to proteolytic breakdown. This is further supported by the partial absence of the surface ruffles on partially degraded HIVHBc preparations. This same disposition was also suggested for the FMDV VP1 chimera based on the appearance of immune complexes (Clarke *et al.*, 1987).

The appearance and size of the HBcHIV particles suggested that the HIV oligopeptide was densely packed in the particle interior. A model for 27-nm particles, based on sequence similarity to picornaviral capsid proteins, proposed that both the N- and C-termini are interior to the particle (Argos and Fuller, 1988). Also, an interior disposition of the C-terminus has been previously inferred based on the presence of a highly basic "protamine"-like tract in the C-terminal one third of the core protein, which is believed to be involved in nucleic acid binding. A core protein mutant with this domain deleted still assembled into a particulate structure, but failed to bind HBV nucleic acids (Gallina *et al.*, 1989; Birnbaum and Nassal, 1990). While some C-terminal fusions in another study had an opaque appearance similar to ours, one (involving 48 amino acids of bovine leukemia virus gp51) clearly did not (Borisova *et al.*, 1989). Also, antibody to this peptide labelled with immunogold formed a halo about the surface of these particles, suggesting their availability at the surface. The reason for this apparent discrepancy is not known. More recently, a peptide derived from the HBsAg pre-S1 region was found to be not disposed at the particles surface when fused to the HBcAg N-terminus, while the same peptide was available at the surface when fused the N-terminus via a short HBcAg precore-derived peptide linker (Schodel *et al.*, 1992). Thus, it may be that structural characteristics of the fused peptide sequences are more important to their disposition than the particular terminus at which they are fused.

Antisera raised against both the N-terminal and C-terminal fusions were able to immunoprecipitate the non-glycosylated form of HIV-1 gp120 protein. This suggests that linear epitopes within the HIV oligopeptides elicited antibodies directed against the non-glycosylated gp120 backbone. Furthermore, antisera to non-glycosylated gp120 immunoprecipitated both chimeric fusion particles. A similar cross-reactivity profile was not seen by Western blot assay. Anti-chimeric particle sera was not able to recognize non-glycosylated gp120 (53 kDa), and anti-53 kDa sera was not able to recognize chimeric particles. Presumably the epitopes recognized by immunoprecipitation were denatured beyond recognition during the blotting procedure, or the assay was not sufficiently sensitive (although very high concentrations of sera were attempted).

Antisera against HIVHBc chimeric particles did recognize virion p17 by Western blot, but not by immunoprecipitation. This suggests that this serum recognizes a p17 epitope revealed after denaturation. The reactivity of anti-HIVHBc, but not anti-HBcHIV serum, to p17 on a Western blot is curious. In contrast to the gp120 oligopeptides, the p17 peptide on the HBcHIV preparation (administered in Freund's adjuvant) might still be sequestered from the immune system inside the particle interior. Alternatively, the C-terminal fusion oligopeptide might adopt a conformation different to that of the N-terminal fusion oligopeptide, eliciting antisera with a different reactivity to blotted p17.

We have demonstrated the successful application of HBcAg fusion particles involving multiple fused foreign peptides. This should be advantageous in several regards. Multiple peptides would allow the selection of epitopes able to stimulate different arms of the immune system where known (e.g. T_h and T_c cell determinants). This would be especially important in the case where involvement of the cellular immune system is thought necessary for elimination of pathogen. Also, the use of multiple epitopes would maximize the chances of targeting corresponding structural features with inflexible functional characteristics, and minimize the likelihood of mutational escape. These features might be ideal in the case of HIV-1, which can exist in the absence of viremia, is thought to spread by cell fusion, and is highly mutable. Some drawbacks of peptide

immunogens might still be operative in this system, such as the failure to elicit neutralizing antibodies due to conformational differences with native virion proteins, or a corresponding structural feature which is not accessible. However, the selection of an appropriate peptide repertoire should overcome these limitations. Further experimentation is necessary to prove that chimeric particles similar to ours are fully reactive to AIDS patients' sera, and capable of eliciting protective immunity.

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