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Immunogenicity of recombinant core particles of hepatitis B virus containing epitopes of human immunodeficiency virus 1 core antigen

Brief Report

R. Ulrich[1], G. P. Borisova[2], E. Gren[2], I. Berzin[2], P. Pumpen[2], R. Eckert[4], V. Ose[3], H. Siakkou[1], E. J. Gren[2], R. von Baehr[4], and D. H. Krüger[1]

Institutes of [1] Virology and [4] Immunology, Charité, Berlin, Federal Republic of Germany
[2] Institute of Molecular Biology and [3] A. Kirchenstein Institute of Microbiology, Latvian Academy of Sciences, Riga, Latvia

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Summary. A Gag protein segment of human immunodeficiency virus 1 (HIV-1) has been fused to a C terminally truncated core antigen of hepatitis B virus (HBcAg) using an *E. coli* expression system. Fusion of 90 amino acids of HIV-1 Gag protein to HBcAg still allowed the formation of capsids presenting on their surface epitopes of HIV-1 core protein, whereas fusion of 317, 189, or 100 amino acids of Gag prevented self-assembly of chimeric particles. Mice immunized with recombinant particles emulsified with Freund's complete adjuvant (CFA) or aluminium hydroxide developed high anti-HBcAg titers. However, anti-HIVp24 antibodies were detected only in mice inoculated with immunogen emulsified with CFA.

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The expression of the core gene of the hepatitis B virus (HBcAg) in *E. coli* [5, 21] and the self-assembly of capsids which differ only slightly from authentic capsids in shape, size [8] and immunological respect [16] have been shown earlier. HBcAg is one of the favoured carriers for the presentation of foreign antigenic determinants. In addition to the high-level synthesis of recombinant particles in *E. coli* there are important advantages of HBcAg as a carrier: HBcAg can serve as a T-cell and B-cell immunogen and shows a high efficiency of T-cell priming [17]. Furthermore, one could expect recombinant capsids, compared with peptides, to induce higher antibody titers, as was shown by Francis et al. [10] for human rhinovirus (HRV) VP 2 peptide.

In the core gene of hepatitis B virus (HBV) sites have been localized which allow the insertion and presentation of foreign antigenic determinants on the surface of expressed chimeric particles [2, 3, 6, 7, 20, 22]. Furthermore, it was shown that deletion of the arginine-rich C-terminus of HBcAg does not impair its ability to form capsids in *E. coli* [2, 11, 22]. Up to 50 foreign amino acids were inserted into the C-terminus of HBcAg without influencing capsid morphogenesis [3, 22].

In order to estimate the maximum packaging capacity of HBcAg at the C-terminus we introduced parts of the Gag protein sequence (p17, p24, p15) of the human immunodeficiency virus 1 (HIV-1) which is crucial for the diagnosis of HIV-1 infections [9]. Using the HBcAg fusion protein containing 90 amino acids (aa) of HIV-1 Gag we investigated the presentation of the inserted epitopes on the particle and compared its immunogenicity in mice with 2 different adjuvants.

In order to express HBcAg-HIV (Gag) fusion proteins we constructed the expression plasmid pHIV24-5 by subcloning a large fragment of the *gag* gene of HIV-$1_{BH\,10}$ [19] encoding 12 C-terminal aa of p17, the complete p24 (231 aa) and 74 N-terminal aa of p15 into the vector pHBc 5 (G. Borisova, unpubl.) (see Fig. 1).

Using the plasmid pHIV24-5 we constructed 3 plasmids expressing Gag protein sequences of different lengths (Fig. 1):

(*i*) To obtain plasmid pHIV24-del1 plasmid pHIV24-5 was digested with *Hind* III, treated with Klenow polymerase and religated.

(*ii*) For further shortening pHIV24-5 was partially digested with *Sph* I, religated and transformed into RR1 (*sup*E44 *hsd*S20 (*res*B *mod*B) *ara*-14 *pro* A 2 *lac*Y 1 *gal* K2 *rps* L 20 *xyl*-5 *mtl*-1) cells. A DNA mixture was prepared from approximately 10^4 cells, cleaved with *Apa* I and, without ligation, retransformed into RR1. The plasmid which expresses a HBcAg fusion protein containing 12 aa of p17 and 88 aa of p24 was designated pHIV24-del2.

(*iii*) The third variant was made by *Pst* I cleavage of pHIV24-5, religation and transformation (pHIV24-del3A). To restore a stop codon immediately after *Pst* I site pHIV24-del 3 A was digested with *Hind* III, treated with Klenow polymerase, religated and transformed into RR 1 cells (pHIV24-del 3).

For expression the recombinant plasmids were retransformed into *E. coli* K 12 strain K 802 (*sup* E *hsd* R *gal met* B), cultivated and induced as described earlier [3]. Total lysates of *E. coli* cells were prepared by boiling cells in Laemmli buffer [13]. The expression of different-sized HBcAg-HIV (Gag) fusion proteins was demonstrated by Western blots using monoclonal antibodies directed against HBcAg (not shown) or monoclonal antibodies 4-1-1 (Fig. 2) and 13-5 (not shown) directed against HIV-1 p24 [12]. The epitopes recognized by the anti-p24 monoclonal antibodies and spanning aa 173 to aa 191 and aa 152 to aa 161, respectively, were identified by the use of synthetic peptides (H. Döpel, unpubl. results) (numbering of aa according to [19]).

Lysozyme lysates of *E. coli* recombinants carrying plasmids pHIV24-5,

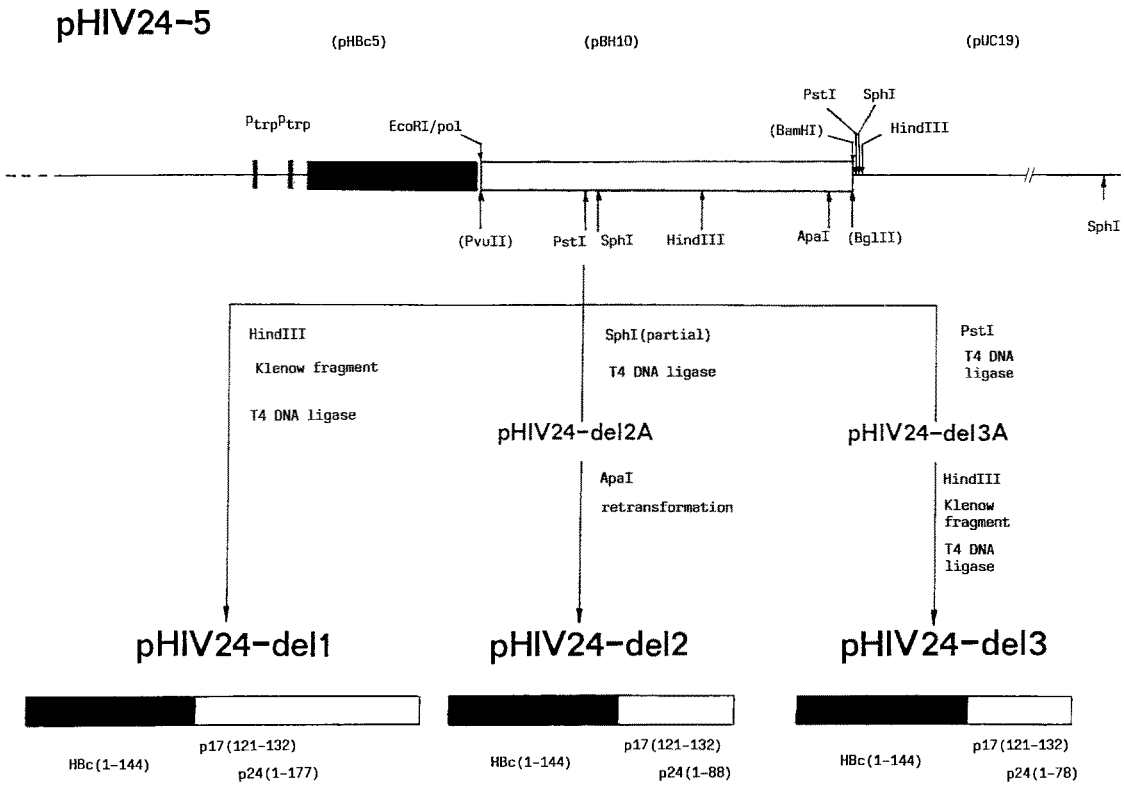

Fig. 1. Structure of the plasmid pHIV24-5 and construction scheme of plasmids pHIV24-del 1, pHIV24-del 2, and pHIV 24-del 3 encoding HBcAg-HIV (Gag) fusion proteins. Recombinant plasmids were constructed by standard techniques [14] on the basis of vector pHBc 5 (G. Borisova, unpubl.) which contains a deleted HBcAg gene controlled by the *trp* promoter and lacks the coding information for the 39 C-terminal amino acids. It has an optimized translation initiation region and an *Eco* RI site for inserting foreign DNA into the codon for the 144th amino acid of HBcAg. The *gag* gene segment was isolated from plasmid pING3 [25] containing an *Eco* RI-*Bgl* II fragment from pBH10 clone [19]. For the expression of a HBcAg-p24 fusion protein a *Pvu* II fragment of pING3 was inserted into pHBc 5 cleaved with *Eco* RI and treated with Klenow polymerase to generate pHIV 24-5. Three deletion variants of pHIV24-5 were constructed, pHIV24-del 1, pHIV24-del 2, and pHIV24-del 3. The DNA sequences of recombinant plasmids were verified by restriction mapping and dideoxy sequencing

pHIV 24-del 1, pHIV24-del 2, or pHIV24-del 3, respectively, were analysed in the Ouchterlony double diffusion test [18] against human sera containing anti-HBc and anti-HBe antibodies. In all cases HBe activity was observed while only the shortest variant pHIV24-del 3 also possessed HBc activity. From controlled titrations (dilution 1 : 128) an expression rate of approximately 33% of the total cellular protein was estimated.

To study the presentation of the inserted Gag peptide, the fusion protein expressed by pHIV24-del 3 was purified by sepharose C14B chromatography. It was recovered from fractions indicative of multimeric structures. Direct evidence for the capsid-forming ability of the fusion protein (containing 90 amino acids

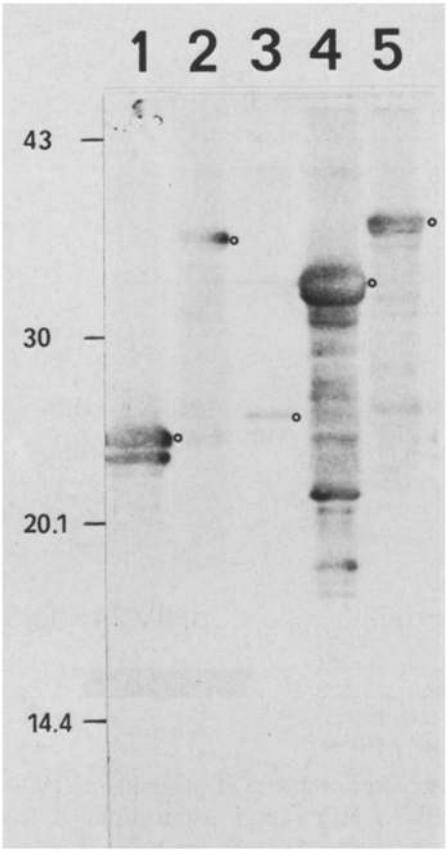

Fig. 2. Detection of expression of HBcAg-p24 fusion proteins by Western blot using anti-p24 monoclonal antibody 4-1-1 [12]. Lysates of *E. coli* recombinants pHIV24-del 3 (*1*), pHIV24-del 3 A (*2*), pHIV24-del 2 (*3*), pHIV24-del 1 (*4*), and pHIV24-5 (*5*) were subjected to gel electrophoresis. The proteins were transferred to nitrocellulose filters as described [23]. Molecular weight markers on the left (in kDa): ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1) and α-lactalbumin (14.4). ○ Migration of the HBcAg fusion proteins

of HIV1-Gag) was provided by electron microscopy (Fig. 3). Furthermore, the surface localization of p24 epitopes was confirmed by ELISA. Chimeric capsids formed by pHIV 24-del 3 were recognized by anti-HBc antibodies which are directed against conformational epitopes [4] as well as by monoclonal anti-p24 antibodies 4-1-1 and 13-5 (not shown).

In contrast to these data the HBcAg fusion protein containing 100 foreign amino acids of HIV-1 Gag was expressed in approximately the same range but did not assemble to capsids. Therefore, it is suggested that the insert length allowing capsid formation is limited to approximately 90 amino acids in recombinant HBcAg lacking the Arg-rich C-terminus, resulting in a similar size as HBcAg recombinants containing both a 50 aa insert and the C-terminus [3, 24].

The described large packaging capacity offers the possibility of investigating the simultaneous insertion of different short epitopes onto the surface of one carrier molecule. The capacity of the N-terminal and e1 loop structures in HBcAg for foreign epitopes is smaller [7, 7 a]. On the other hand, Ty virus-like particles were reported to even tolerate the insertion of 213 aa of HIV-1 Env protein [1].

To analyze immunogenicity we inoculated mice of 3 different strains (XVII, Balb/c, AB/Bln) with two doses of the recombinant HBcAg-HIV (Gag) particles using as adjuvants either complete Freund's adjuvant (CFA) or aluminium hy-

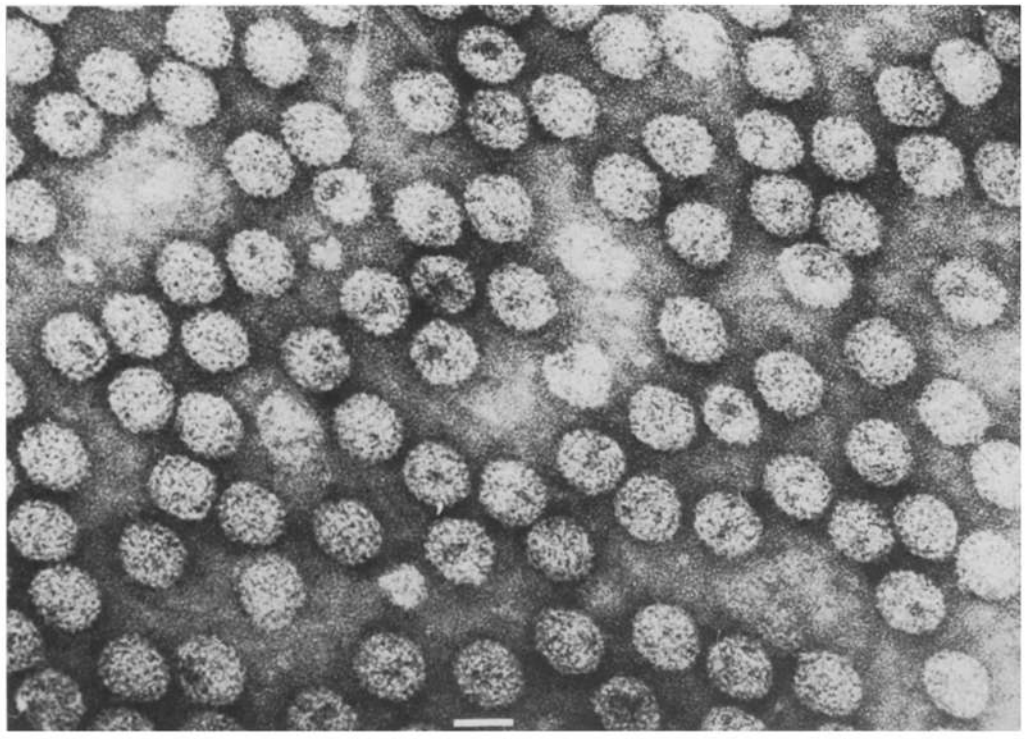

Fig. 3. Electron microscopic image of purified HBcAg-HIV (Gag) capsids expressed by pHIV24-del 3 (× 400,000). Samples for electron microscopic investigations were prepared as described earlier [3]. Bar: 25 nm

droxide (Table 1). In a first experiment male mice of strains XVII, Balb/c and AB/Bln (5 animals per group) were subcutaneously inoculated 3 times with 100 μg of the chimeric HBcAg-Gag particles formulated in CFA. Booster injections were done at days 28 and 42. In a second experiment mice of strain AB/Bln (4 animals per group) were immunized with 100 μg or 500 μg purified HBcAg-Gag particles in aluminium hydroxide by three subcutaneous injections at 1 month intervals.

The immunogen emulsified in CFA induced a strong anti-HBc response in all animals already detectable on the seventh day and after only one inoculation. The anti-HBc titer increased significantly after the second inoculation (1/40,000) whereas the third inoculation had only little effect. The three mice strains tested did not differ significantly.

When the immunogen was administered as an aluminium hydroxide precipitate HBc-specific antibodies were detectable only after the second inoculation, then reaching the same titers as with CFA. Titers of anti-HBc antibodies were in the same range as published by others [10, 20] independent of the adjuvant used. Anti-HIV titers in the murine sera were estimated by an indirect ELISA with disrupted virions. Anti-HIV antibodies were detectable after the 2nd inoculation of the immunogen and only with the immunogen emulsified in CFA.

Table 1. Response of different mice strains to HBcAg-HIV (Gag) particles

Strain	Dose	Adj.	EIA-Ag	Days after initial inoculation					
				0	7	14	36	50	65
XVII	100	CFA	HBcAg	<1[a]	3.7	nt	4.7	4.8	nt
			HIV	<1	<1	nt	2.6	2.9	nt
Balb/c	100	CFA	HBcAg	<1	3.5	nt	4.3	4.6	nt
			HIV	<1	<1	nt	2.9	3.4	nt
AB/Bln	100	CFA	HBcAg	<1	3.9	nt	4.1	4.3	nt
			HIV	<1	<1	nt	2.5	3.2	nt
AB/Bln	100	$Al(OH)_3$	HBcAg	<1	nt	<1	4.2	nt	4.3
			HIV	<1	nt	<1	<1	nt	<1
AB/Bln	500	$Al(OH)_3$	HBcAg	<1	nt	<1	4.0	nt	4.0
			HIV	<1	nt	<1	<1	nt	<1

Serum samples obtained before immunization and seven days after each inoculation were serially diluted and tested for HBcAg or HIV-p24 specific antibodies by commercial ELISAs (Institute of Molecular Biology, Riga; Sächsisches Serumwerk, Dresden). Titers are expressed as the reciprocal of the serum dilution giving an absorbance value three times higher than with preimmune sera

[a] $\log_{10}$ mean EIA endpoint titer

nt Not tested

Compared with anti-HBc the titers of HIV-specific antibodies were generally lower by at least one order of magnitude (range 1/500–1/2,500).

Similar results have been reported for fusions with HBsAg [15], to the N-terminus [7, 10] and C-terminus of HBcAg [3, 20] expressed in different host organisms. To explain the decreased immunogenicity of carboxyterminally-fused sequences it was speculated that only one in three or less HBc molecules flip to expose their carboxyl-terminus to the outside of core particles, thus achieving a lower epitope density per particle [20]. The loss of immune response against HIV1-Gag after complexing the particles with aluminium hydroxide remains unclear. In similar experiments with HBcAg-HRV2-VP 2 peptide complexed with aluminium hydroxide the antibody titers were also low [10].

Currently there are two problems posed by recombinant carrier molecules: maximal insert length and optimal sites for insertion allowing surface presentation and maximal immunogenicity. Until recently the maximal length of inserts was reported to be approximately 50 aa [3]. We have shown that it is possible to fuse 90 foreign aa to the C terminally truncated HBcAg, still being compatible with the formation of chimeric capsids. Further experiments are needed to investigate whether this high packaging capacity of HBcAg is restricted to Gag protein sequences or is also relevant for Env sequences which are of main importance for vaccine development.

The discrepancy between the titers of antibodies directed against HBcAg and HIVp24 (described here) or against other inserted epitopes [3, 10, 20] regardless

of insertion into the N- or C-terminus of HBcAg remains unclear. Further experiments are required to define whether the presentation of foreign epitopes is only a statistical phenomenon or is structurally determined and predictable. Recently, improved immunogenic properties of chimeric particles have been reported after insertion of epitopes into the e1 loop structure of HBcAg [19 a].

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Authors' address: R. Ulrich, Institute of Virology, Charité, Schumannstrasse 20/21, D-1040 Berlin, Federal Republic of Germany.

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