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# Design of yeast-secreted albumin derivatives for human therapy: Biological and antiviral properties of a serum albumin–CD4 genetic conjugate

(albumin conjugates/*Kluyveromyces*/plasma clearance/therapeutic protein design)

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ABSTRACT **Due to its remarkably long half-life, together with its wide *in vivo* distribution and its lack of enzymatic or immunological functions, human serum albumin (HSA) represents an optimal carrier for therapeutic peptides/proteins aimed at interacting with cellular or molecular components of the vascular and interstitial compartments. As an example, we designed a genetically engineered HSA–CD4 hybrid aimed at specifically blocking the entry of the human immunodeficiency virus into CD4$^+$ cells. In contrast with CD4, HSA–CD4 is correctly processed and efficiently secreted by *Kluyveromyces* yeasts. In addition, its CD4 moiety exhibits binding and antiviral *in vitro* properties similar to those of soluble CD4. Finally, the elimination half-life of HSA–CD4 in a rabbit experimental model is comparable to that of control HSA and 140-fold higher than that of soluble CD4. These results indicate that the genetic fusion of bioactive peptides to HSA is a plausible approach toward the design and recovery of secreted therapeutic HSA derivatives with appropriate pharmacokinetic properties.**

In addition to the existing need for recombinant natural proteins useful for human therapy, the design and production of hybrid proteins combining particular biological properties of their initial components has great potential. For example, biologically active polypeptides/proteins often exhibit a rapid *in vivo* clearance, requiring significant amounts of material to achieve efficient concentrations during therapy. Furthermore, small polypeptides with molecular masses below the 20-kDa range have been reported to be readily filtered at the level of the renal tubules (glomerulus), often leading to a dose-dependent nephrotoxicity. Therefore, the fusion of unstable biological polypeptides/proteins to a large suitable carrier exhibiting high *in vivo* stability, together with the development of an efficient expression system, are particularly relevant to the design of protein-derived pharmaceuticals.

Human serum albumin (HSA) is widely distributed throughout the body, in particular in the interstitial and blood compartments where it is mainly involved, as the most abundant protein of the serum (40 g per liter, ≈0.7 mM), in the maintenance of osmolarity. Furthermore, HSA is slowly cleared by the liver and displays an *in vivo* half-life of several weeks (1). Importantly, HSA is devoid of any enzymatic or immunological function and, thus, should not exhibit undesired side effects after coupling to a bioactive polypeptide. In addition, HSA is a natural carrier involved in the endogenous transport and delivery of numerous natural as well as therapeutic molecules (2). Altogether, these particular features make HSA an optimal candidate for the carrier of biologically active peptides/proteins in both vascular and extravascular interstitial compartments.

Chemical cross-linking of porcine growth hormone to serum albumins has been reported and resulted in interesting modifications of the pharmacokinetics of the growth hormone–albumin conjugates. This modification included a 20- to 40-fold increase in stability as compared with uncoupled growth hormone in a rat experimental model, as well as an altered pattern of tissue distribution, strongly suggesting clearance through the liver and reduced chance of nephrotoxicity (3). However, as pointed out by the authors, the "error nature of the cross-linking procedure" raises important limitations concerning exact formulation and reliability of such pharmaceutical preparations, a problem that should be avoidable by using genetic engineering techniques such that a composite gene encoding a suitable HSA conjugate can be secreted and easily recovered in a homogeneous state.

Despite the natural structural complexity of HSA—a naturally secreted unglycosylated protein (molecular mass, 66 kDa) the globular structure of which is maintained by 17 disulfide bonds—*Kluyveromyces* yeasts can secrete several grams per liter of a recombinant HSA (rHSA) indistinguishable from its natural counterpart (4). We further widened the usefulness of this microbiological secretion system to the design and production of HSA derivatives displaying other biological properties. As an example, we designed a genetic yeast-secreted HSA conjugate aimed at blocking the binding of the human immunodeficiency virus (HIV) to its target cells.

Recombinant soluble CD4 (sCD4) blocks the binding and penetration of HIV into CD4$^+$ target cells and is nontoxic *in vivo*. However, sCD4 is rapidly cleared in humans, making it difficult to reach efficient clinical concentrations (5, 6). It is, therefore, particularly important to design CD4 conjugates with an improved half-life. In addition, the recent report that primary HIV isolates might be less efficiently blocked by sCD4 than laboratory strains (7) emphasizes the need to use doses higher than initially suggested by *in vitro* studies. An efficient expression system is, thus, an absolute requirement to achieve this goal. In this report we show that the *Kluyveromyces* expression system can efficiently secrete a genetic

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Abbreviations: HSA, human serum albumin; HIV, human immunodeficiency virus; sCD4, soluble CD4; mAb, monoclonal antibody; rHSA, recombinant HSA.

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HSA–CD4 conjugate exhibiting the desired biological properties of each initial constituent.

## MATERIALS AND METHODS

**Construction of Expression Plasmids.** Expression vectors were pKD1 (8, 9) derivatives that replicate in *Kluyveromyces* yeasts (10). An *Mst* II–*Hin*dIII fragment corresponding to the V1V2 domains of CD4 was generated by PCR with the CEM13 cell line as a source of CD4 mRNA. As primers 5′-CCCGGGAAGCTTCCTTAGGCTTAAAGAAAGTGGTGCTGGGCAAAAAAGGG-3′ and 5′-CCCGGGAAGCTTTTAGAAAGCTAGCACCACGATGTCTAT-3′ were used. The amplified fragment was fused with HSA at the *Mst* II site located 4 residues upstream from the C-terminal end, engineering a hybrid between complete HSA and the first 179 residues of CD4 (plasmid pYG365B) (Fig. 1*A*). The plasmid pYG221B is the same construct lacking the CD4 fragment.

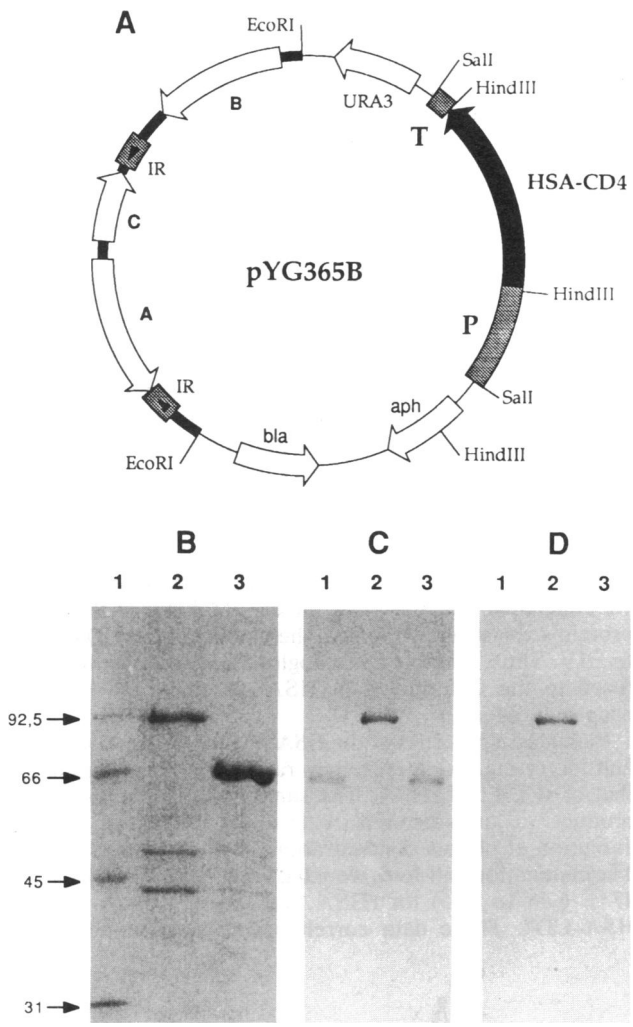


FIG. 1. *Kluyveromyces* HSA–CD4 secretion system. (*A*) Map of expression plasmid pYG365B. P, promoter; T, transcription terminator; IR, pKD1 inverted repeats. Genes A, B, and C of pKD1 and the phenotypic markers are indicated. (*B*) Coomassie blue staining after electrophoretic migration of supernatants in an 8.5% polyacrylamide gel. Molecular mass standards (lane 1); supernatants of pYG365B (HSA–CD4, lane 2) or pYG221B (rHSA, lane 3) transformants are shown. (*C*) Immunoblot analysis of supernatants, as revealed by a polyclonal serum directed against HSA; standard HSA (lane 1) and supernatants of *Kluyveromyces* yeasts transformed by plasmid pYG365B (lane 2) or pYG221B (lane 3) are shown. (*D*) Immunoblot as for *C*, except that a polyclonal serum directed against CD4 was used.

**Immunoblotting.** After SDS/PAGE, samples were transferred to a nitrocellulose membrane (0.45-μm pore size, Shleicher & Schuell) by semidry electrotransfer (Bioblock Scientific, Illkirsh, France) at 1 mA/cm$^2$ for 30 min. The filter was first incubated with specific rabbit serum and then incubated with a biotinylated goat anti-rabbit IgG serum followed by an avidin–peroxidase complex (Vectastain-ABC kit, Biosys, Compiègne, France).

**Purification of the Hybrid Protein.** The procedure used will be detailed elsewhere. Briefly, after 60 hr of culture, the medium is centrifuged, and the pretreated supernatant is loaded on a QMA-Spherosil column (IBF). The flow-through fraction is then passed onto a pseudo-affinity Fractogel TSK AF-Red column (Merck). Finally, the eluate is chromatographed on a Q-Sepharose fast-flow column (Pharmacia). Fusion proteins with a purity of at least 95% were obtained.

**Immunological Characterization.** ELISA plates coated with HSA–CD4 (0.1 μg per well) were incubated with $3.2 \times 10^{-11}$ M of Leu3a monoclonal antibody (mAb) (Becton Dickinson) or OKT4A mAb (Ortho Diagnostics) preincubated with increased amounts of sCD4 (11) or HSA–CD4. Bound mAb was revealed with a peroxidase-linked goat anti-mouse IgG serum (Biosys). Absorbance at 600 nm was measured after addition of 3,3′,5,5′-tetramethylbenzidine.

***In Vitro* Binding to gp160.** ELISA plates were coated with sCD4 (50 ng per well) and incubated with 125 fmol of gp160 (12) preincubated with increased amounts of sCD4, rHSA, or HSA–CD4. The residual binding of gp160 to coated-CD4 was revealed by the successive addition of an anti-gp160 mouse mAb (110.4, Genetic Systems, Seattle), followed by a peroxidase-linked goat anti-mouse IgG serum. Absorbance at 492 nm was measured after addition of *o*-phenyldialenine.

**Cell Culture.** The cells were maintained in RPMI 1640 medium/10% fetal calf serum/2 mM L-glutamine/penicillin at 50 units/ml and streptomycin at 50 μg/ml.

**Binding of HIV Particles to CD4$^+$ Cells.** Cells ($5 \times 10^5$) were incubated with 2 μg of heat-inactivated HIV-$1_{BRU}$ particles initially preincubated with 116 pmol of either HSA–CD4 (10.7 μg), rHSA (7.5 μg), or sCD4 (5 μg). Residual binding of the viral particles to the cells (13) was determined after successive incubations with anti-gp160 110.4 mAb and a phycoerythrin-labeled goat anti-mouse IgG serum; fluorescence was measured with a cell sorter. The negative control is the lymphoblastic CEM13 cell line incubated with the two antibodies.

**HIV Infection of CD4$^+$ Cells.** HSA–CD4 and sCD4 were first incubated in microtiter wells with HIV-1 infectious cell-free supernatants (25 tissue culture 50% infective dose) in 100 μl of fresh medium. CEM13 cells ($10^4$) were then added, and the plates were incubated for 1 hr at 37°C and centrifuged; the supernatants were then carefully removed. Two hundred fifty microliters of fresh medium containing HSA–CD4 or sCD4 was then added, and the plates were further incubated for 4 days. p24 antigen determination (p24-ELISA DuPont) and cell viability were assayed (14). Fifty microliters of the remaining cells was transferred to another plate containing 200 μl of fresh medium supplemented with HSA–CD4 or sCD4. Incubation was continued until day 7, and the same procedure was repeated on days 11, 14, 18, and 21 after infection to establish the long-term efficacy of the compounds.

**Cell Viability Assay.** The experiment was done as described (14). Ten microliters of (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at 5 mg/ml was added to each well, and plates were incubated for 4 hr at 37°C. One hundred fifty microliters of a 2-propanol/0.04 M HCl mixture was then added, and the formazan crystals were resuspended. Absorbance at 540 nm was measured.

***In Vivo* Half-Life Experiments.** At least two male New Zealand White (Hy/Cr) rabbits were used for each product.

They were kept under constant temperature, lighting, and humidity conditions. The same molar quantity of each product was administered in a single injection in the marginal vein of the ear: sCD4 (250 μg), rHSA (400 μg), or HSA–CD4 (500 μg). Three-milliliter blood samples were taken before injection ($t_o$), then 5 min, 10 min, 20 min, and 30 min, 1 hr, 2 hr, 4 hr, and 8 hr after injection from rabbits injected with sCD4, or at $t_o$, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 24 hr, 32 hr, 48 hr, 56 hr, 72 hr, 80 hr, 96 hr, 104 hr, and 168 hr from rabbits injected with HSA–CD4 or rHSA. The samples were mixed with lithium heparinate, centrifuged, divided into three aliquots, and assayed by an ELISA method. Assays of sCD4 were done on ELISA plates coated with HSA–CD4. Increased concentrations of sCD4 or the samples to be assayed were incubated with the OKT4A mouse mAb (dilution 1:1000). Residual binding of OKT4A was revealed by a peroxidase-linked anti-mouse IgG serum (Nordic). For assaying rHSA, the plates were initially coated with an anti-HSA serum (Sigma A0659, dilution 1:1000). Increased concentrations of HSA or the samples to be measured were then added, followed by a peroxidase-linked anti-HSA serum (Nordic). HSA–CD4 was measured, either by assaying the HSA moiety as for rHSA or by a bifunctional assay: ELISA plates were coated with an anti-HSA serum and then incubated with the samples to be assayed. Leu3a mAb was then added, followed by the peroxidase-linked anti-mouse IgG serum. For each experiment, absorbance was measured after addition of 2,2′-azino-bis(3-ethylbenzothialozine-6-sulfonic acid)diammonium salt (11557 Fluka).

## RESULTS

**Design and Secretion of HSA–CD4 in *Kluyveromyces*.** The CD4 surface antigen is composed of four extracellular immunoglobulin-like domains designated V1–V4 (15). The most N-terminal domain of the antigen, V1, binds the HIV gp120 with high affinity, although the V2 domain somehow contributes to optimal binding (16–18). Recent x-ray crystallography data demonstrated that both domains are actually closely packed together (19, 20). In addition, because anti-CD4 auto-antibodies have been found in HIV-infected patients after injection of the V1V4 version of sCD4, but not after injection with immunoadhesins containing only the V1V2 domains (21, 22, 24, ¶), only these two domains were fused to HSA. To minimize the effect of the fusion on secretion efficiency of the hybrid protein, the V1V2 domains were fused to the C terminus of HSA. Culture supernatants were submitted to electrophoresis and the gels were stained with Coomassie blue. Fig. 1*B* shows that *Kluyveromyces* yeasts transformed with the HSA–CD4 construct secrete a protein of ≈90-kDa molecular mass. This unglycosylated protein is by far the most abundant protein in the supernatant because it can represent up to 70% of total proteins as monitored by densitometry scanning (data not shown). Immunoblot analysis with polyclonal anti-HSA (Fig. 1*C*) or anti-CD4 (Fig. 1*D*) serum demonstrates that the HSA–CD4 protein is recognized by both antisera. Gram quantities of HSA–CD4 and rHSA were purified from the transformed *Kluyveromyces* cell-culture supernatants. N-terminal sequencing of both recombinant proteins revealed the N-terminal sequence of mature albumin (Asp-Ala-His . . .), demonstrating that the prepro sequence of HSA is functional and correctly processed after the pair of basic amino acids $Arg^{-2}$-$Arg^{-1}$. This correct maturation is likely from the *KEX1* convertase of *Kluyveromyces lactis* (25).

¶Yarchoan, R., Pluda, J. M., Adamo, D., Thomas, R. V., Mordenti, J., Goldspiel, B. R., Ammann, A. J. & Broder, S., *Sixth International Conference on AIDS*, June 20–24, 1990, San Francisco, abstr. S.B. 479.

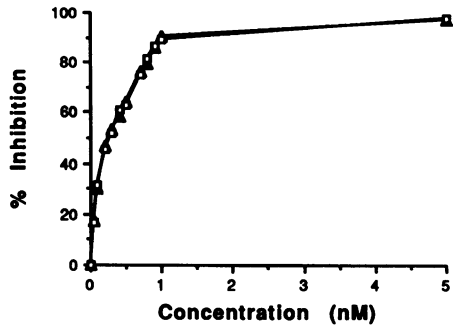


FIG. 2. mAb Leu3a binding to HSA–CD4 and sCD4. ▲, CD4; □, HSA–CD4.

**Binding and Antiviral Properties of the CD4 Moiety.** In an initial attempt to assess HSA–CD4 bioreactivity, we used an ELISA competition test (26) to characterize its equilibrium dissociation constants ($K_d$) for mouse mAbs OKT4A (data not shown) and Leu3a (Fig. 2), both directed against epitopes proximal to the gp120-binding site of the CD4 V1 domain. For mAb OKT4A, the $K_d$ values were $3.1 \times 10^{-9}$ M for sCD4 and $3.7 \times 10^{-9}$ M for HSA–CD4; for mAb Leu3a they were $2.6 \times 10^{-10}$ M and $2.7 \times 10^{-10}$ M for sCD4 and HSA–CD4, respectively. Therefore, sCD4 and HSA–CD4 exhibit identical immunological reactivities toward both mAbs.

We also examined the ability of HSA–CD4 to block the interaction between HIV and its receptor. Increased concentrations of HSA–CD4 block the monovalent interaction of gp160 with coated-sCD4 in a dose-dependent manner. On a molar basis this blocking efficiency is similar to that of sCD4 (Fig. 3). As measured by fluorescein-activated cell sorter (FACS) analysis, HSA–CD4 also inhibits the multivalent gp160–CD4 interaction occurring between heat-inactivated HIV particles and $CD4^+$ CEM13 cells (Fig. 4). Finally, preincubation of infectious HIV particles with increased amounts of sCD4 or HSA–CD4 leads to a similar dose-dependent inhibition of viral p24 production, demonstrating that both products inhibit viral infection of susceptible human T cells to the same extent (Fig. 5). From these experiments we concluded that the HSA moiety (66 kDa) of the hybrid protein does not interfere with the binding of the CD4 moiety to HIV. Thus, variable immunoglobulin-like domains can be fused to the C terminus of HSA, so that both parts are properly folded.

**Biological Properties of the HSA Moiety.** We measured the half-life of the fusion protein in rabbit and compared it with that of sCD4 and rHSA. The same molar quantity of each product was administered i.v. in a single injection, and the evolution of plasma concentrations was monitored (Fig. 6). The elimination half-lives were 0.25 ± 0.1 hr ($n$ = 4) for sCD4, 47 ± 6 hr ($n$ = 5) for rHSA, and 34 ± 4 hr ($n$ = 5) for HSA–CD4. These data correlate with a clearance of ≈3

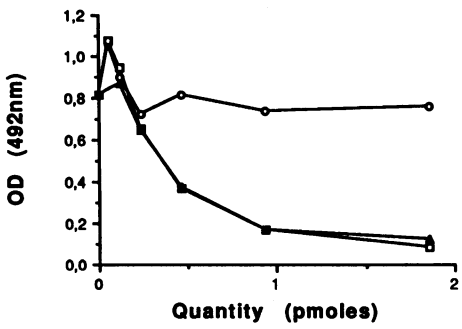


FIG. 3. Inhibition of sCD4 binding to gp160. ▲, CD4; □, HSA–CD4; and ○, HSA.

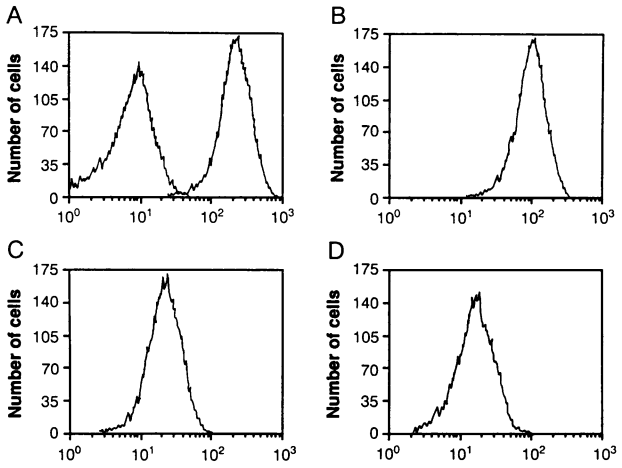


FIG. 4. Inhibition of binding of HIV particles to CD4$^+$ CEM13 cells. (*A*) Negative control (left) and HIV-1 alone (right). (*B*) HIV-1 plus HSA. (*C*) HIV-1 plus CD4. (*D*) HIV-1 plus HSA–CD4.

ml/min per kg for sCD4 compared with ≈0.02 ml/min per kg for rHSA and HSA–CD4. For HSA–CD4, similar values were obtained with ELISAs specific for the HSA and the CD4 moieties. Therefore, HSA–CD4 exhibits an elimination half-life in rabbit 140-fold higher than that of sCD4 and comparable to that of rHSA.

## DISCUSSION

Previous studies revealed that chemical cross-linking to serum albumins could be used to enhance the *in vivo* half-life of otherwise unstable polypeptides (3, 27). For superoxide dismutase, this enhancement was particularly important because half-life was increased from 4 min to 6 hr after i.v. administration (28). However, chemical coupling usually leads to a complex of heterogeneous nature, and it also requires finding appropriate cross-linking conditions so that the conjugate will retain the biological properties of both constituents. In that regard, a genetic coupling is preferred, and our results suggest that HSA can be used as a carrier for bioactive peptides characterized by poor pharmacokinetic properties. In particular, our HSA–CD4 genetic conjugate can be efficiently secreted in yeasts, it retains the antiviral properties of its CD4 moiety, and its half-life has been improved 140-fold as compared with that of sCD4 in a rabbit experimental model.

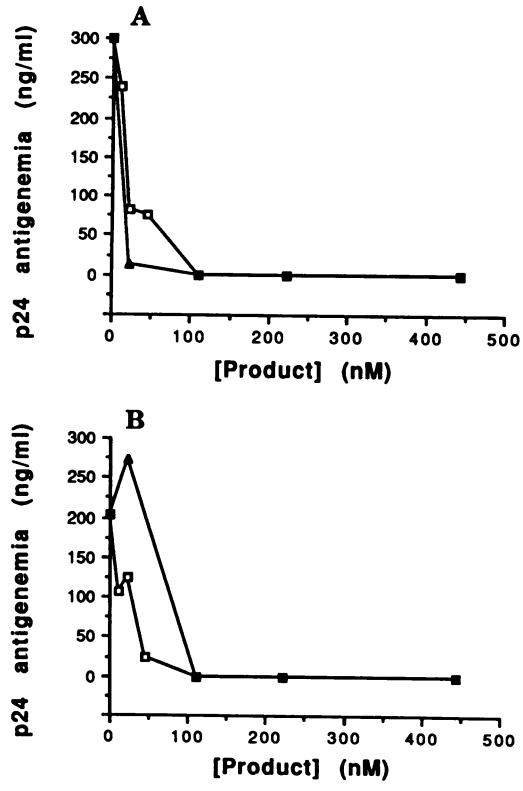


FIG. 5. Inhibition of HIV replication in CD4$^+$ CEM13 cells. ▲, sCD4; □, HSA–CD4. (*A*) Day 7. (*B*) Day 11.

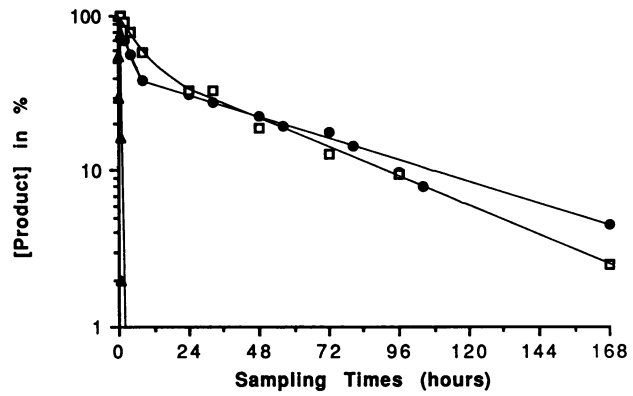


FIG. 6. Evolution of the plasma concentrations of sCD4, rHSA, and HSA–CD4 in rabbits. ▲, sCD4; □, HSA–CD4; and ●, rHSA.

HIV induces a progressive, life-threatening disease in every infected individual, and so far no efficient therapy has been found. Although numerous drugs acting at various steps of the HIV infectious cycle have been shown to be potent HIV inhibitors *in vitro*, only a few have yet been used in humans. Among them, inhibitors of reverse transcriptase are being extensively studied in clinical trials. For example, 3′-azido-3′-deoxythymidine (AZT) is very potent *in vitro* and is currently the most clinically active drug. However, its usefulness is hampered by a severe toxicity *in vivo* and the appearance of HIV escape mutants invariably arising after a few months of treatment (29). So far, other nucleoside analogs also display toxicity, and it is not yet clear whether their use in combination therapy will improve their clinical efficiency. This problem can probably be better approached by using nucleoside analogs in combination with drugs acting at other steps of the HIV cycle. Interestingly, sCD4 acts synergistically with AZT *in vitro* (30) and is, thus, a potential therapeutic drug to be used in such multi-target therapeutic schemes. However, efficient clinical concentrations of sCD4 have not been achieved, presumably because of its rapid clearance *in vivo* (5, 6). The design of appropriate CD4 conjugates with an improved half-life is, therefore, crucial.

Given the immunoglobulin-like structure of CD4, the fusion of the CD4 V1V2 domains to various immunoglobulin heavy chains (collectively referred to as immunoadhesins) was, in part, aimed at such an effect. These composite molecules were produced from mammalian cell cultures and demonstrated a similar, or even better, *in vitro* capacity to block HIV infection as compared with sCD4 (31–33). A phase I clinical trial with one such molecule showed no significant toxicity and a 3- to 10-fold increase in half-life as compared with that of sCD4 (23, 24). In spite of this enhancement and although partially effective therapeutic concentrations might have been reached, this treatment did not result in significant changes in CD4 lymphocyte counts of p24 antigen levels in serum (24).

Immunoadhesins do not interact solely with the CD4-mediated entrance of the HIV-1 particles because they retain a functional Fc fragment displaying beneficial properties for

the treatment of HIV-infected patients, including the mediation of antibody-dependent cell-mediated cytotoxicity toward infected cells and placental transfer (33). However, low titers of HIV-specific antibodies have been reported to significantly enhance infection of several human cell lines *in vitro*, including cells of the monocyte lineage, fibroblasts, lymphocytes, or lymphoblastic cell lines. This antibody-dependent enhancement of infection apparently occurred after binding of the Fc fragment of the antibodies either directly to Fc receptors (34–39) or indirectly to complement receptors (40–42). Although the *in vivo* significance of the antibody-dependent enhancement phenomenon is unclear for lentiviruses, the presence of a functional Fc fragment in the immunoadhesins raises the possibility that HIV spreading might occur through a CD4-independent pathway. Stable CD4 conjugates, such as the HSA–CD4 reported here, aimed at interacting solely with the virus particles should, therefore, also be studied. In addition, because HSA–CD4 and sCD4 exhibit similar antiviral properties in every assay we used and because HSA–CD4 and the V1V2 immunoadhesin display a similar half-life improvement as compared with that of sCD4 in the same animal model (31), the HSA–CD4 hybrid actually represents a valid alternative to the immunoadhesins.

It is also important that the HSA–CD4 hybrid is correctly matured and efficiently secreted by several wild-type strains of *Kluyveromyces* yeasts. This microbiological expression system also represents a significant improvement for producing CD4 conjugates to be used in rather large therapeutic doses. This fact is of particular interest because a recent observation suggested that primary HIV isolates might be less efficiently blocked by sCD4 *in vitro* than laboratory strains (7). High concentrations of HSA–CD4 should be well tolerated in patients because HSA is an abundant neutral plasma protein and sCD4 does not interact with major histocompatibility complex class II molecules (23). Animal studies together with clinical trials will further address this question.

In a more general sense, HSA might also be considered as a secretion-competent carrier in yeast. This is true, at least for CD4, because a construct isogenic to plasmid pYG365B (HSA–V1V2), but lacking the mature HSA sequence, yielded very poor secretion levels of the V1V2 protein and in a mostly degraded form as indicated with a polyclonal anti-CD4 serum (P.Y., unpublished results).

Finally, our results also indicate that efficient secretion of suitable HSA genetic conjugates in *Kluyveromyces* yeasts is compatible with separate refolding of the two parts of the fusion protein and that hybrid proteins can be engineered so that the HSA moiety will not hinder the biological activity of the polypeptide linked to it.

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