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THE USE OF RECOMBINANT PROTEINS IN AIDS RESEARCH: DEVELOPMENT OF A CD4/GP120 BINDING ASSAY

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INTRODUCTION

The continuous increase in the number of people infected with the Human Immunodeficiency Virus (HIV), the causative agent of the acquired immunodeficiency syndrome, AIDS, mandates the development of effective strategies to stop the threatening pandemic. Recombinant DNA technology is of crucial importance not only to increase our knowledge of the biology of the AIDS virus, but also to allow the rapid development of accurate diagnostic tools, of new therapeutic approaches and protective vaccines. The availability of large amounts of recombinant proteins opens new perspectives in all areas of applied AIDS research, as illustrated by the following example.

The first step in the HIV infection cycle involves the binding of the viral envelope glycoprotein, gp120, to the cell-surface receptor, CD4, present on helper T-lymphocytes and monocyte/macrophages (1). Because of the high affinity interaction between gp120 and CD4 is required for infection with all strains of HIV, various therapeutic approaches based on this interaction have been proposed. One strategy has been to identify anti-CD4 mAb that mimic the binding site on gp120 for CD4 and use these as idiotypic vaccines (2-4). Another has been to identify the gp120 binding site on CD4 and design synthetic peptides which mimic the binding domain (5). A third, more promising approach has been to express soluble forms of the CD4 protein (6-10) or molecular fusions of CD4 with either toxins (11,12) or immunoglobulins (13,14) that block HIV infection and selectively kill HIV-infected cells. Although this strategy represents a potential antiviral therapy for HIV infection, it requires large amounts of recombinant protein to treat one patient; this may cause manufacturing problems. In addition, the therapy of AIDS patients is expected to be longlasting. The injection of large doses of recombinant soluble CD4 over extended periods could induce antibodies that neutralize the antiviral effect.

As an alternative approach we designed a gp120/CD4 binding assay using recombinant proteins. This system allows high-flux screening for low molecular weight, preferentially oral-bioavailable compounds that interfere with the binding of HIV to the target cell.

MATERIAL AND METHODS

Plasmid constructs and microbiological manipulations

A BamHI-HindIII cDNA fragment encoding the first two domains (V1+V2) of human CD4 was isolated from plasmid pHCD4 (kindly provided by A. Traunecker, Institute for Immunology, Basel) by introducing a BamHI restriction site at position aa+1 after the signal peptide cleavage site by site directed mutagenesis and by ligating a HindIII recognition sequence to the NheI site immediately upstream of the V2 splice junction. The resulting DNA fragment was then inserted into the E.coli expression vector pDS56/RBSII,6xHis (15). The CD4 C-terminal deletion mutants were made by Bal 31 exonuclease digestion from the HindIII site, followed by Klenow polymerase treatment and ligation of a HindIII linker. Expression of all recombinant CD4 proteins was in the E.coli strain M15 harbouring the lac repressor-producing plasmid pDMI,1 as described previously (16).

Purification of recombinant CD4

CD4, V1+V2 and all deletion mutants were expressed with a hexa-histidine affinity label at the amino terminus. This permits a rapid purification of mg quantities from a crude lysate by metal chelate affinity chromatography (15,17). Cultures of the recombinant bacteria were grown in antibiotic-supplemented L-broth until A_{600} ~0.7, after which ß-D-thiogalactopyranoside was added to a final concentration of 400 µg/ml. After an incubation period of 4h, the bacteria were collected by centrifugation and the pellets lysed in 6M guanidine hydrochloride, pH 8.0. The purification of 6His-CD4 over nickel nitrilotriacetate-Sepharose was then essentially done as described by Stüber et al. (15). CD4 bound to the column was eluted at pH6, renatured by dialysis against 10 mM Na-acetate pH 6.5 and stored frozen at -80°C. By this method 75% pure, soluble CD4 could be recovered in one step from a crude bacterial lysate with a yield of 20-30 mg/l.

Construction of recombinant baculoviruses

The baculovirus transfer vector pVL941, obtained from M. Summers, Department of Entomology, Texas A&M University, was used to express HIV-1 gp120. From the HIV-1 proviral clone HAN2 (18) a AvaII to BsmI restriction fragment encoding gp120

was tagged with BamHI linkers and inserted into the BamHI site of pVL941 to generate pVL-gp120. Cotransfection of this plasmid together with wild type Autographa californica nuclear polyhedrosis virus DNA into Spodoptera frugiperda (Sf9) cells was by lipofection (Gibco). Recombinant occlusion negative viruses were isolated by limiting dilution followed by plaque purification on agar plates (19). Recombinant gp120 secreted into the culture medium of 200 ml spinner cultures of Sf9 cells infected with recombinant baculovirus was purified as described previously (20,21).

Immunological assays

The binding affinity of recombinant CD4 to the anti-CD4 mAbs Leu-3a (Becton-Dickinson) and OKT4a (Ortho), and the interaction of recombinant gp120 with recombinant CD4 were assayed by ELISA essentially as described by Gallati et al. (22).

Virological assay

The antiviral activity of the different recombinant proteins was measured in a syncytium formation assay. In short, a pretitrated amount of HIV-1, HAN (18) was incubated at room temperature with serially diluted recombinant proteins for 30 min. Thereafter, 25 µl of each mixture was transferred in triplicate into the wells of a 96-well plate which contained 25,000 MT-2 cells (23) per well in 50 µl of medium. After 3 days of culture, 100 µl of fresh medium was added and after 5 days the syncytia were counted in each well.

RESULTS AND DISCUSSION

Expression of soluble CD4 in E.coli

As firstly demonstrated by Traunecker et al. (10) the immunoglobulin-like and second domains of human CD4 are sufficient for binding the HIV envelope protein gp120 and to protect target cells against HIV infection. Therefore we introduced a cDNA fragment encoding the first two domains (V1+V2) into the E.coli expression vector pDS56/RBSII,6xHis (15) which directs the production of heterologous proteins N-terminally tagged with a hexa-histidine tail. This strategy allows the rapid purification of recombinant proteins by affinity

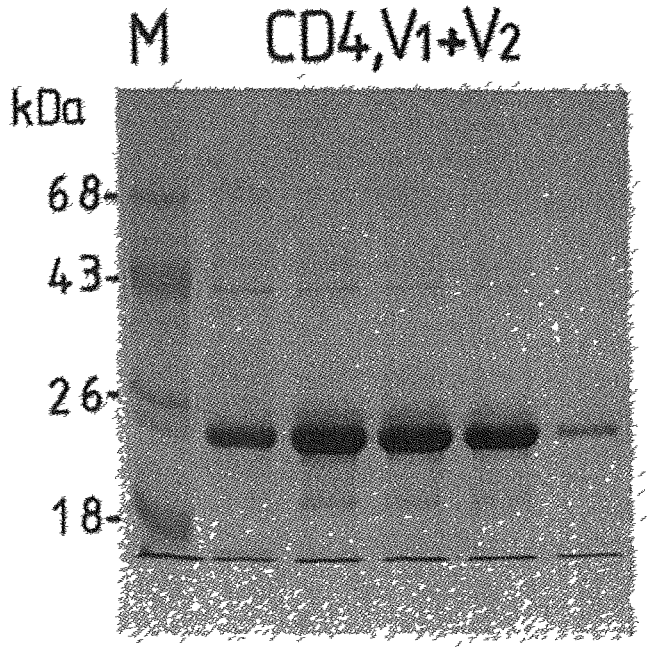

Fig. 1. SDS-PAGE analysis of recombinant CD4 present in the peak fractions eluted at pH6.0 from the NTA-column (see Materials and Methods). Proteins are visualised by Coommassie Blue staining. Lane M represents molecular weight standard proteins.

chromatography over a Ni-chelate column as described in Material and Methods. As shown in Figure 1 the recombinant CD4,V1+V2 eluted from the column was around 75% pure after a single purification step from the crude bacterial lysate. Next, the peak fractions of the eluate were pooled and directly dialysed against 10 mM Na-acetate, pH6.5. This simple method was found to give the highest yields of soluble CD4. Moreover, this CD4 preparation bound with high affinity to the mAbs Leu-3a and OKT4a, which recognize part of the gp120 binding domain on native CD4 (24), when measured in an ELISA-type assay (data not shown). This result demonstrates that our recombinant CD4,V1+V2 expressed in E.coli adopted the proper configuration to generate biological activity (which will be described later) in spite of the presence of artificial amino acids at the N- and C-terminus (see Fig.2).

Serial C-terminal deletions of CD4, V1+V2

Affinity purified soluble CD4 containing the domains V1 and V2 was tested for antiviral activity in a HIV-1 dependent syncytium formation assay using MT-2 cells

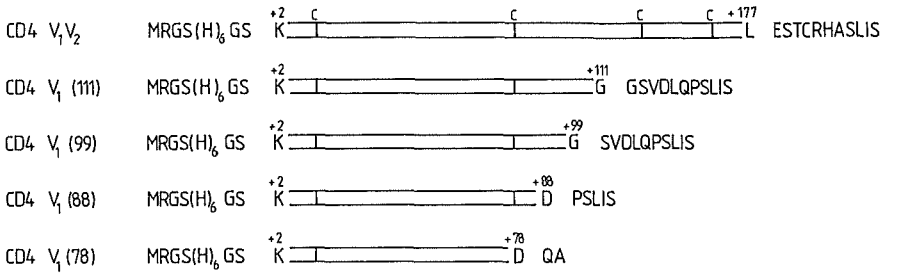

Fig. 2. Primary sequence of the different CD4 variants expressed in E.coli. One letter amino acid abbreviations are used.

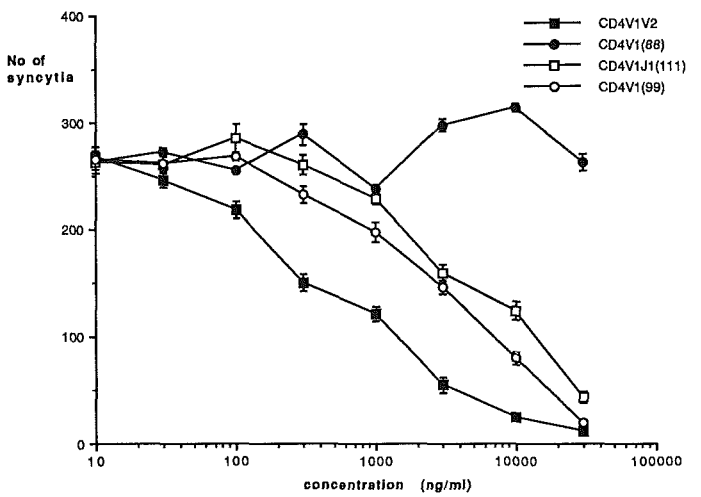

Fig. 3. Analysis of the antiviral activity of different recombinant CD4 molecules in a HIV-1 syncytium assay (see Materials and Methods) . The indicated values are means of triplicate assays.

as outlined in the Material and Methods section. HIV-1 infection of MT-2 cells resulted in the formation of typical balloon-type cells (syncytia), on average 260 syncytia per well of a microtiter plate after 5 days of incubation. As illustrated in Figure 3, CD4,V1+V2 inhibited the occurrence of syncytia by 50% at a concentration of 0.8 µg/ml (IC_{50} = 0.8 µg/ml), which approximates the results published in the literature (6-9).

In order to determine the minimal length of soluble CD4 required for the observed antiviral effect, we made serial 3'-terminal deletions of the CD4 DNA insert by Bal31 exonuclease treatment as indicated in the Methods section. The expected amino acid sequences of the different CD4 proteins expressed from the mutant gene fragments is depicted in Figure 2. Note that the various CD4 mutants contain the same artificial N-terminal sequence, including the hexa-His label to allow a standard affinity purification over a Ni-chelate column. The primary sequence of the CD4 protein was as described by Mizukami et al. (25). After purification of the different recombinant CD4 variants to the same extent as described for CD4,V1+V2, the protein preparations were tested for their capacity to block HIV infection. The results of these experiments are shown in Figure 3. Both CD4 mutants truncated at position 111 and 99 of the primary sequence still retained the potency to inhibit HIV-infection. However, the mutant V1 (88) containing CD4 sequences up to amino acid position 88 completely lost the antiviral activity. The same result was obtained with mutant V1 (78) (data not shown). Concomitant with the loss of antiviral activity, mutants V1 (88) and V1 (78) also lost the reactivity with the mAbs Leu-3a and OKT4a.

Production of recombinant gp120 in insect cells

Relatively large amounts of the HIV-1 envelope glycoprotein gp120 can be produced when using the baculovirus expression system (20). Sf9 cells infected with recombinant baculovirus containing the gene for HIV-1,HAN gp120 (18) under the control of the polyhedrin promoter secrete ± 5 µg/ml gp120 into the culture medium at day 4 after infection (L. Dirckx, unpublished observation).

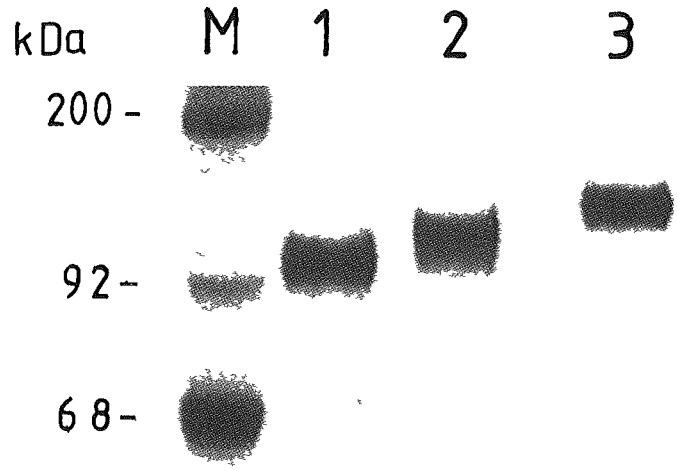

Fig. 4. Gel electrophoretic analysis of envelope glycoprotein gp120 immunoprecipitated from the culture medium (lane 1) or the cell lysate (lane 2) of Sf9 cells infected with recombinant baculovirus, and, for comparison, from the medium of HIV-1 infected JURKAT cells. Immunoprecipitations with a HIV^{+}-serum were done as described previously (20).

As can be seen in Figure 4, the HIV envelope protein produced in insect cells is somewhat smaller than authentic gp120 produced in HIV-1 infected human T-cells. This is due to differences in glycosylation (20). Nevertheless, the recombinant gp120 was found to have the same high affinity (Kd~3.10^{-9}M) for the CD4 receptor as reported for the native envelope protein (21).

A prototype CD4/gp120 binding assay

The availability of mg amounts of both ligand (gp120) and receptor (CD4) allowed us to establish a screening assay for compounds that can interfere with the CD4/gp120 interaction. CD4,V1+V2 was purified from a bacterial lysate as described in Materials and Methods. Recombinant gp120 was isolated from a 200 ml culture of infected Sf9 cells by CD4-affinity purification (21). After coating microtiter plates with 10 µg/ml CD4,V1+V2 in 0.1M sodium hydrogen carbonate, pH8.0 (22), the different wells were incubated with increasing concentrations of

purified gp120 in sample dilution buffer (22) for 1h at 37°C. After 2 washing steps with PBS/0.05% Tween-20, a 1:1000 dilution of HIV+-serum in PBS/Tween/20% FCS was added and incubated overnight at 4°C. Finally, extensive washing with PBS/Tween was followed by a 1h incubation at room temperature in the presence

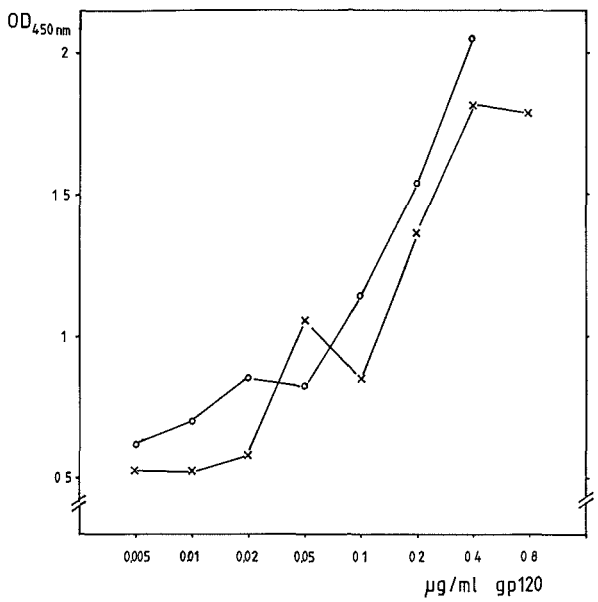

Fig. 5. Titration of recombinant gp120 on CD4,V1+V2-coated microtiter plates by ELISA as described in the text. O-O and X-X represent two different batches of purified gp120.

of conjugate (goat anti-human IgG-peroxidase conjugate, 1:1000). Peroxidase activity was then measured by addition of 5.5'-tetramethylbenzidine (22). The results of these experiments are illustrated in Figure 5. As can be seen, recombinant gp120 interacted with CD4,V1+V2 in a dose-dependent way, showing saturation at a concentration of approximately 0.8 µg/ml. Therefore, a concentration of 1 µg/ml was used in our prototype CD4/gp120 binding assay, which can be used to identify novel compounds that may block the high affinity interaction of the HIV virus with its taret cells. Further improvements of this basic screening system are underway. The final goal is a high-flux, direct binding assay using recombinant CD4,V1+V2 and peroxidase-conjugated gp120 in a single step enzymatic immunoassay.

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Discussion -THE USE OF RECOMBINANT PROTEINS IN AIDS RESEARCH: DEVELOPMENT OF A CD4/GP120 BINDING ASSAY

H.J. Röthig

What about oral availability of the Roche protease inhibitor?

J. Mous

There is some oral bioavailability observed in rats and marmosets. It is not yet known whether the compound is orally bioavailable in man.

A. Ganser

What kind of toxicity can be expected or anticipated for the protease inhibitor?

J. Mous

The HIV protease belongs to the class of aspartic proteinases as also the human enzymes pepsin, renin and cathepsin. However, our protease inhibitor displays a very high specificity and has practically no inhibitory effect on the human enzymes. Furthermore the inhibitor shows a very large therapeutic index, because of its high antiviral potency and low cytotoxicity. Preliminar experiments in animals do not show any acute toxicities from treatment with the compound at relatively high doses. However, we have to await the results of the ongoing toxicity studies to give a more precise answer.

R.G. Werner

Have you done a stoichiometric calculation of how many CD4 molecules you would need to inactivate one virus and how many grams of CD4 constructs you would need for one dose, and how much material you would need for the entire treatment of the life cycle of the patient?

J. Mous

There is one problem in that calculations done in the laboratory are mostly done with laboratory strains of HIV. So the calculations based on these viruses are probably not reflecting the real situation in patients. In fact, to prevent clinical isolates to infect T-cells in vitro one needs up to a thousand fold more soluble CD4 than to prevent the infection by a laboratory strain HIV. And the first results of treatment of patients with soluble CD4 seem to reflect that situation because these trials do not show any effect of treatment on any of the parameters of viremia in the treated patients. There is no

drop in p24 and there is no increase of CD4 cells. This is not due to antibody neutralization of injected soluble CD4. It seems that in spite of the high doses used in these trials, the amount of protein is still not high enough to efficiently prevent the virus to infect the cell. These viruses are quite different from the ones used in the laboratory.