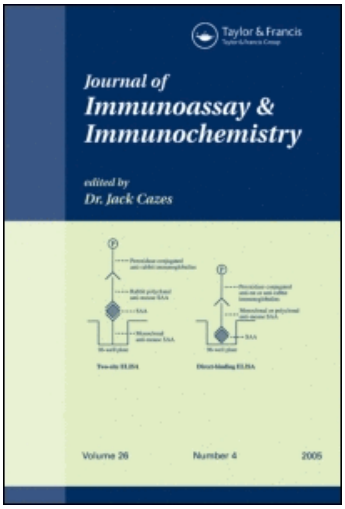

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development and Characterization of a Whole-Cell Radioligand Binding Assay for [^{125}I] gp120 OF HIV-1

L. P. To[ab]; V. Balasubramanian[b]; M. E. Charlton[c]; T. A. Francis[d]; C. Doyle[e]; P. M. Sweetnam[b]
[a] Department of Biology, Goucher College Towson, Maryland, USA [b] Nova Screen®, A Division of Nova Pharmaceutical Corp, Maryland, USA [c] Department of Biology, Georgetown University Washington, District of Columbia, USA [d] Department of Biomolecular Discovery, Smith Kline Beecham Pharmaceuticals, Pennsylvania, USA [e] Division of Immunology, Duke University Medical Center, Durham, North Carolina, USA

To cite this Article To, L. P. , Balasubramanian, V. , Charlton, M. E. , Francis, T. A. , Doyle, C. and Sweetnam, P. M.(1992) 'Development and Characterization of a Whole-Cell Radioligand Binding Assay for [^{125}I] gp120 OF HIV-1', Journal of Immunoassay and Immunochemistry, 13: 1, 61 — 83
To link to this Article: DOI: 10.1080/15321819208019825
URL: http://dx.doi.org/10.1080/15321819208019825

JOURNAL OF IMMUNOASSAY, 13(1), 61-83 (1992)

DEVELOPMENT AND CHARACTERIZATION OF A WHOLE-CELL RADIOLIGAND BINDING ASSAY FOR [^{125}I]gp120 OF HIV-1

L.P. To (1,2), V. Balasubramanian (2), M.E. Charlton (3), T.A. Francis (4) C. Doyle (5), and P.M. Sweetnam (2)

(1) Present address: Department of Biology, Goucher College Towson, Maryland 21204 USA
(2) NovaScreen®, A Division of Nova Pharmaceutical Corp. 6200 Freeport Centre, Baltimore, Maryland 21224 USA
(3) Department of Biology, Georgetown University Washington, District of Columbia, USA
(4) Department of Biomolecular Discovery, SmithKline Beecham Pharmaceuticals King of Prussia, Pennsylvania USA
(5) Division of Immunology, Duke University Medical Center Durham, North Carolina USA

ABSTRACT

The binding of HIV-1 envelope glycoprotein, gp120, to the CD4 receptor is an important step in productive infection. The development of agents which interrupt this binding phenomenon should be of therapeutic interest. The present study characterizes a whole cell gp120/CD4 radioligand binding assay (radioligand binding assay) modified for use in a high volume screening format. Modifications include the use of human CD4 receptor stably expressed in a Chinese hamster ovary cell line and the gentle fixation (paraformaldehyde) of the CD4 receptor just prior to assay. Binding of [^{125}I]gp120 to fixed CD4 was of high affinity (K_D = 6 nM), saturable, reversible, and specific. The kinetics of binding were identical to those of viable (non-fixed) CD4 receptor. [^{125}I]gp120 binding was inhibited by unlabeled recombinant gp120, soluble CD4, and the anti-CD4 monoclonals OKT4A and LEU3A. A number of compounds reported to inhibit gp120 binding and/or gp120 induced syncytium formation were also active in this assay. This modified radioligand binding assay was developed to initiate a rational and extensive screening program to assist in the identification of potential chemotherapeutic agents based on their ability to inhibit gp120 binding to host cells. (KEY WORDS: Radioligand Binding Assay, gp120, HIV)

INTRODUCTION

The first step in the multiplication of many animal viruses is the attachment of virions to receptors on the plasma membrane of a target cell. Such receptors may be integral glycoproteins which exhibit a high degree of affinity for some aspect of the envelope structure of the invading virus. In the case of Human Immunodeficiency Virus type-1 (HIV-1) one route of infectivity is initiated by the binding of gp120, the viral envelope glycoprotein, to the CD4 receptor, a host cell membrane glycoprotein. Therefore, T cells, B cells and cells of monocyte/macrophage lineage which express the CD4 receptor, appear to be preferential targets for HIV-1 infectivity.

The CD4 molecule's capacity to bind to gp120 and the ability of anti-human CD4 antibodies and soluble CD4 peptides to block HIV-1 infectivity *in vitro* (1-8), suggests this step in the HIV-1 replicative cycle could be exploited in the development of chemotherapeutic agents. Several previously identified compounds which inhibit gp120/CD4 binding and gp120 induced syncytium formation *in vitro* have not yet been proven effective chemotherapeutic agents *in vivo*. One class of compounds, the sulfated polysaccharides, e.g., dextran, heparin and pentosan sulfate (9), interfere with blood coagulation and may lack binding selectivity. Another approach using soluble CD4 or related peptide analogues which coat the HIV-1 virus (10-12), thereby masking the viral particle may, after repeated treatment, trigger an immune response possibly generating antibodies which compromise CD4 receptor function.

The identification of molecules which inhibit gp120 binding to the CD4 receptor but in themselves have no intrinsic bioactivity, would be useful in AIDS therapy. Such compounds, termed gp120 antagonists, can initially be identified by screening chemical libraries using a gp120/CD4 radioligand binding assay. Compounds discovered in this manner would, in principle, serve as templates for the development of gp120 antagonists which could interfere with HIV-1 absorption by the target cells. Furthermore, such antagonists could be utilized as biological probes to dissect the function of the CD4 receptor.

Using iodinated gp120 as a ligand and human CD4 stably expressed in a chinese hamster ovary (CHO) cell line, we have developed a radioligand binding assay (13-14) which eliminates many of the technical problems associated with previously described gp120/CD4 binding assays (15-17). First, the number of CD4 receptors present on the cell lines used in these studies was relatively low (<100,000 receptors per cell) resulting in low signal to noise ratio. Second, the use of viable cells often results in assay to assay variability which is unacceptable in a high volume screening assay. By eliminating these technical problems we have been able to initiate a rational and extensive screening program to assist in the identification of potential chemotherapeutic agents based on their ability to inhibit gp120 binding to host cells.

MATERIALS AND METHODS

Recombinant soluble CD4 consisting of the entire extracellular domain of the receptor was a generous gift from Dr. Tak Mak and

J. Schiller (University of Toronto). A recombinant form of gp120(SKB), prepared using a drosophila expression system, was provided by SmithKline Beechman Pharmaceuticals. A second recombinant gp120(ABT), prepared using a baculovirus expression system, was purchased from American Biotechnologies, Inc. Bolton-Hunter reagent was purchased from NEN/Dupont and PD-10-column from Pharmacia. Peptide T, peptide T-amide, and gp120 peptide fragment (amino acids 254-274) was purchased from Bachem. The anti-CD4 monoclonals OKT4A and LEU3A were obtained from Orthodiagnostics and Becton-Dickinson, respectively. Sheep anti-serum to gp120 and pre-immune sheep serum were obtained through the AIDS research and reference reagent program (Bethesda, MD).

Radioiodination

Radiolabeling of both forms of recombinant gp120 was performed using [^{125}I]Bolton Hunter reagent. This paper describes results obtained using [^{125}I]gp120(SKB). A benzene solution of the [^{125}I]Bolton Hunter reagent was evaporated to dryness using a stream of argon. A charcoal trap was installed to vent any radioactive volatiles. The reagent vial was then cooled to 0°C for five minutes. A 20 μg sample of gp120 in 5 μl of Dulbecco's phosphate buffered saline (DPBS) without Ca^{++} and Mg^{++}, Ph 7.5, was added to the reagent vial and mixed thoroughly. The reaction vial was incubated at 0°C for 2 hours. In the meantime, a PD 10 column (Pharmacia prepacked G25 column) was rinsed two times with DPBS without Ca^{++} and Mg^{++} followed by DPBS without Ca^{++} and Mg^{++} containing 0.2% gelatin

(Gelatin Buffer). After incubation the reaction was quenched by adding 500 μl of 0.2 M glycine buffer (DPBS without Ca^{++} and Mg^{++} containing 0.2 M glycine) and incubating for an additional five minutes. The reaction mixture was then applied onto the PD 10 column and allowed to run through. The reaction vial was rinsed thoroughly with 0.5 ml of gelatin buffer and applied to the column. After this, the vial was rinsed with 1 ml gelatin buffer and added to the column. Following this rinse, the radiolabeled material was eluted with 5 ml of gelatin buffer. Five drops per tube was collected in 24 tubes. The radioactivity in these tubes was measured using Capintec radioisotope calibrator. The radioactivity eluted in fractions 3 to 11 was routinely used in binding studies.

Culture Conditions

Maintenance and CD4 Amplification - A Chinese hamster ovary cell line (CHO/CD4) was co-transfected with the structural genes for the CD4 receptor and the enzyme dihydrofolate reductase (DHFR) (Figure 1). CHO/CD4 and CHO control cells were maintained in Ham's F-12 media supplemented with 10% dialyzed fetal calf serum (Hyclone), 2 mM L-glutamine, 15 mM HEPES, 100 U penicillin, 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air. Cells were split 1:15 weekly. CHO/CD4 cell media was also supplemented with 0.72 μM methotrexate in order to amplify the expression of the CD4 receptor in a stable manner (Figure 1). Two days prior to the assay, cells were detached with 0.02% EDTA at 37°C. Detached cells were pelleted at 1,000 RPM for 5 minutes. The

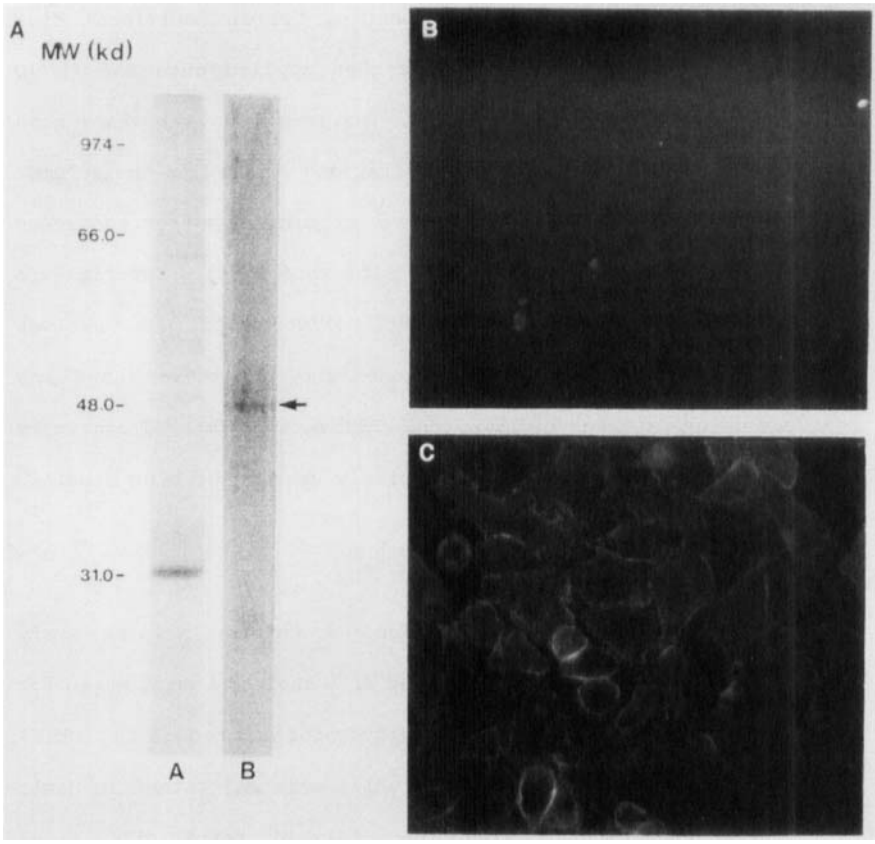

FIGURE 1. CD4 receptor expression was confirmed by (A) Immunoblot of CD4/CHO cell membranes with the anti-CD4 antibodies, LEU-3A. (Lane A) Is a silver stain of total membrane protein, (Lane B) revealed a single immunoreactive protein band with an approximate molecular weight of 50 KDa. Photomicrograph (C) shows CD4-like immunoreactivity following incubation with LEU-3A. Immunoreactivity appears to be associated with cellular membrane. Photomicrograph (B) shows a control culture in which LEU-3A was preabsorbed with soluble CD4.

supernatant was then discarded and cells were resuspended in 0.72 μM methotrexate media at a concentration of 5 x 10^5 cells/ml. Viability was routinely 95% as determined by trypan blue exclusion.

Cells were seeded into 96 well microtitre plates at a density of 5 x 10^4 cells per well and maintained for 24 hours at 37°C. Final cell number per well was approximately 100,000 cells per well yielding a final concentration of 1.6 μg total protein/well at the time of the assay. Only the inner 60 wells of the assay plate were used in order to eliminate variability in cell growth associated in the outer wells of the microtitre plates. Cells were seeded directly on to tissue culture plastic or matrices consisting of CELL TAK, collagen or poly-L-Lysine (Collaborative Research).

Cell Fixation

Cells were washed 3 times for 1 minute with DPBS at room temperature. Cells were then fixed in 50 mM phosphate buffer with 2% paraformaldehyde at room temperature for 30 minutes. The fixative was then removed by extensive washing with DPBS. Cells can be maintained for 24 hours in DPBS at 4°C prior to use in radioligand assay.

Immunochemical Studies

CHO/CD4 cell cultures were fixed by incubating in 50 mM phosphate buffer, pH 7.5, with 4% freshly depolymerized paraformaldehyde for one hour at 4°C. CD4 receptor was localized by incubating fixed cultures with LEU3A, at a dilution of 1:50, in DPBS

at 4°C overnight. The bound antibodies were visualized following incubation with a fluoresceinated goat anti-mouse IgG, at a dilution of 1:40, for 30 minutes at room temperature (Cappel).

CHO/CD4 crude membranes were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). Gels were incubated 45 minutes in transfer buffer (192 mM glycine, 25 mM Tris, pH 8.3, 20% methanol). Proteins were transferred to nitrocellulose by electro-elution (10 mAMPS, overnight). Non-specific binding sites were blocked by incubation of nitrocellulose in 0.05% Tween DPBS for one hour at 27°C. Nitrocellulose was incubated with LEU3A (1:50 in 0.05% Tween DPBS) at 4°C overnight. Sheets were washed with DPBS and incubated with peroxidase labeled goat-antimouse IgG for 2 hours at 27°C. Following several washes CD4 immunoreactivity was visualized using 0.01% diaminobenzidine, 0.1% H_2O_2 in DPBS.

[^{125}I]gp120 Binding Studies

The specific binding of [^{125}I]gp120 was measured using whole cell binding in a microtitre well format. CHO and CHO/CD4 cells were maintained and prepared as described in "Culture Conditions". In the concentration-response assay each well received approximately 1 nM [^{125}I]gp120 (80,000 disintegrations per minute [dpm]) and the appropriate concentrations of either unlabeled gp120 or potential inhibitors. The assay buffer consists of 0.2% DMSO and 5 μg/ml bacitracin PBS pH 7.4; 4°C. Bacitracin was added as a competitive substrate to inhibit possible protease activity in the recombinant gp120 preparations. The final assay volume was 100 μl. Following

an incubation of 60 minutes at 37°C, the assay was terminated by 3 rapid washes with ice cold PBS. After the final wash, wells were treated for 10 minutes at room temperature with 75 μl of 1 N NaOH. A 70 μl aliquot was then transferred to 12 x 75 polypropylene tubes and counted using an LKB gamma counter. In all binding assays non-specific binding was defined as that radioactivity remaining in the presence of 10^{-6} M gp120(ABT) and represented approximately 20% of the total binding. Within individual experiments each data point was generated from the average of triplicate well values. Results were analyzed by a computer-fitting program from Lunden Associates.

Immunoprecipitation of gp120

Sheep anti-gp120 antiserum and formaldehyde-fixed Staphylococcus aureus (Cowan strain) cells were incubated for 45 minutes at 37°C followed by two rinses with PBS. gp120 was then incubated with the above mixture for 45 minutes at 37°C. Immuno-complexed gp120 was then removed by centrifugation (Figure 2).

RESULTS

Characterization of gp120/CD4 Binding

Initial characterization of our CHO/CD4 cell line by immunochemical analysis revealed the CD4 receptor was expressed on the plasma membrane (Figure 1). This receptor also bound gp120 as determined by concentration-response studies in which bound [^{125}I]gp120 was displaced by recombinant preparations of gp120. Both recombinant preparation displaced 80% of total [^{125}I]gp120 bound at

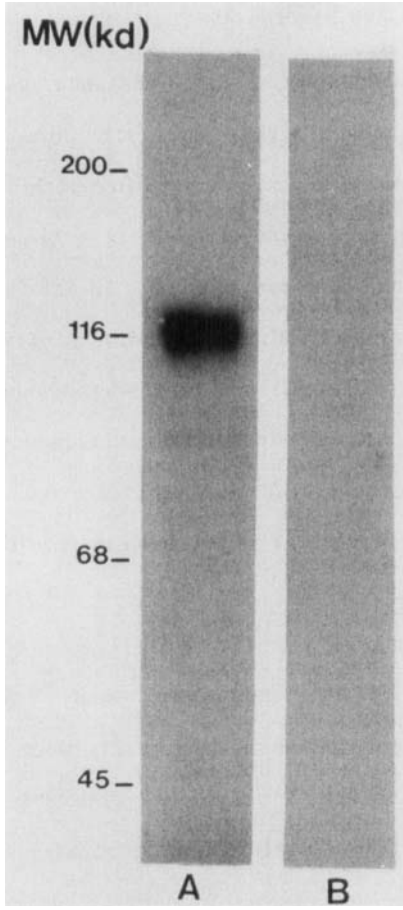

FIGURE 2. The ability of gp120 antiserum to immunoprecipate gp120 was determined by autoradiographic analysis of gp120 preparations run on SDS PAGE. gp120 was tracked by a tracer amount of [^{125}I]gp120. (Lane A) gp120 preparation. (Lane B) gp120 preparation following immunoprecipitation. This preparation was unable to inhibit gp120 binding as determined by radioligand binding assay.

concentrations of 10^{-6} M in fixed cells, K_i = 8 nM gp120(ABT); K_i = 5 nM, gp120(SKB). These studies also examined [^{125}I]gp120 binding in both viable and fixed CHO/CD4 cells and revealed displacement curves generated for both conditions were similar (Figure 3B). Dextran sulfate, a non-selective inhibitor of gp120 binding, also inhibited

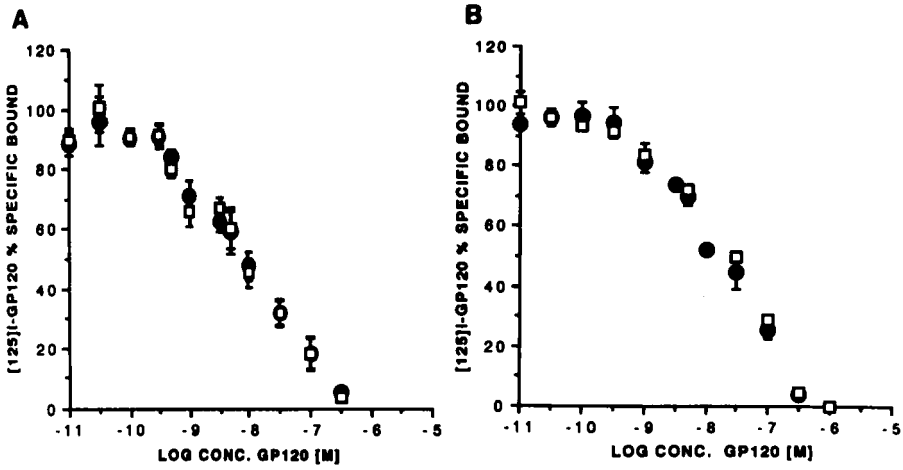

FIGURE 3. (A) Competition inhibition curves of gp120(SKB)(□) and gp120(ABT)(○) of [^{125}I]gp120 binding in CHO/CD4 cells. Cells were incubated with approximately 1 nM [^{125}I]gp120 and various concentrations of unlabeled gp120 as described in Materials and Methods. The data point represents the mean ± SEM of five experiments. (B) Competition analysis of [^{125}I]gp120 binding to viable (•) and fixed (□) CHO/CD4 cells using gp120(ABT). The data are mean values of a representative experiment that was performed in triplicate and replicated a minimum of three times.

approximately 90% [^{125}I]gp120 binding in both viable and fixed cell preparations at a concentration of 10^{-2} M.

The potencies of different compounds which inhibit [^{125}I]gp120 binding was also examined in fixed cells (Table 1). As predicted, soluble CD4 (19) and the anti-CD4 monoclonals OKT4A and LEU3A (20) were potent inhibitors of [^{125}I]gp120 binding: >80% inhibition at nanomolar concentrations. Nanomolar concentrations of aurintricarboxylic acid (21) also inhibited [^{125}I]gp120 >50%. Two other compounds, dextran sulfate and pentosan polysulfate were an

TABLE 1

INHIBITION OF [^{125}I]gp120 BINDING TO FIXED AND FRESH CHO/CD4 CELLS

Compounds	[Conc]	%Inhib.(Fixed)	%Inhib.(Fresh)
gp120 (ABT)	1 uM	100.0 ± 1.4	100.0 ± 12.0
gp120 (SKB)	1 uM	100.0 ± 1.2	100.0 ± 9.0
gp120 (254-274)	100 uM	4.2 ± 2.6	2.0 ± 17.0
Soluble CD4	1 nM	89.7 ± 1.7	81.0 ± 21.0
LEU3A	200 nM	79.0 ± 1.9	-- --
OKT4A	200 nM	62.0 ± 1.9	53.0 ± 14.0
ATCA*	100 nM	81.3 ± 0.5	-- --
Dextran Sulfate	1 nM	89.7 ± 3.0	-- --
Evan's Blue	250 uM	85.8 ± 0.7	-- --
Pentosan PS**	5 mM	70.2 ± 2.3	-- --
Peptide T	10 uM	3.7 ± 1.7	98.0 ± 23.0
Peptide T Amide	10 uM	4.7 ± 3.1	-- --

* - Aurintricarboxylic Acid
** - Pentosan Polysulfate

order of magnitude weaker. In contrast, peptide T, peptide T amide (22), and gp120 peptide fragment (254-274) did not significantly inhibit [^{125}I]gp120 binding.

Results from equilibrium binding experiments (N=4) with fixed receptor revealed that [^{125}I]gp120 apparently binds to a single high affinity CD4 site, (Figure 4) similar to previous results obtained with other cell lines (13-15). Scatchard analysis of this binding revealed a linear plot (Figure 4 inset). Computer-assisted analysis afforded a best fit for a single binding site with an apparent K_D = 6 nM and B_{max} of 68 fMoles (approximately 450,000 receptors per cell). Cold saturation analysis revealed no apparent difference in

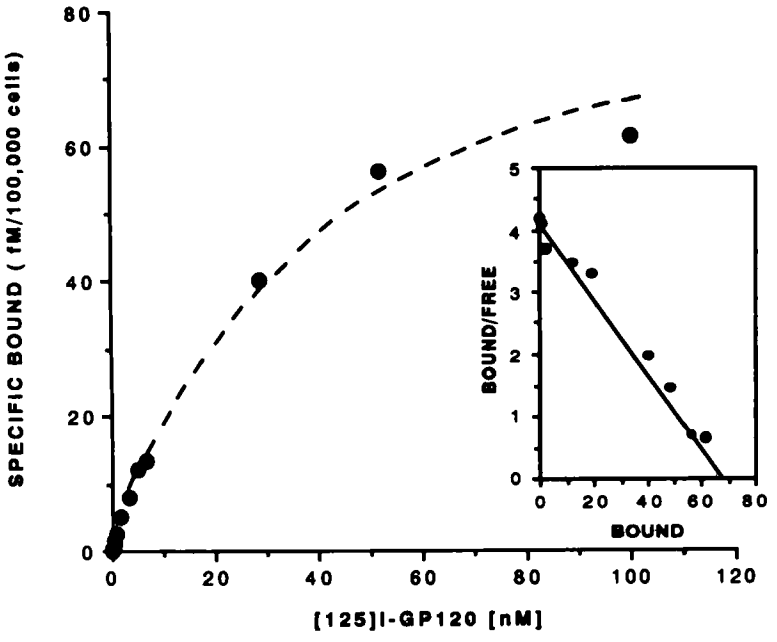

FIGURE 4. Saturation isotherm for the binding of [^{125}I]gp120 to fixed CD4 cells. Cells were prepared and incubated with varying concentrations of [^{125}I]gp120 as described in Materials and Methods. Insert presents Scatchard plot for the data from the saturation isotherm revealed a linear plot (correlation coefficient r = .986) with an apparent K_D = 6 nM and B_{max} of 68 fMoles. These experiments were performed using gp120 (ABT) as cold displacing agent.

either K_D or B_{max} values obtained using viable or fixed cells (data not shown).

[^{125}I]gp120 binding to the paraformaldehyde fixed CD4 receptor expressed in CHO cells was rapid and reversible; it reached equilibrium within 45 minutes at 37°C. Specific binding, determined by [^{125}I]gp120 binding in the presence of 10^{-6} M "cold" gp120(ABT), was approximately 70-80%. In contrast, specific binding was not detectable in an untransfected CHO cell line. With incubation periods of greater than 45 minutes at 37°C, a decrease in specific

binding was noted. The addition of the non-specific protease inhibitor, bacitracin (5 μg/ml), to the incubation buffer stabilized specific binding at >70% for incubation periods in excess of 1 hour at 37°C.

The presence of contaminant proteins in our recombinant gp120 also made it necessary to determine their possible role in the inhibition of [^{125}I]gp120 binding. Proteins remaining after specific immunoprecipitation of gp120 were unable to inhibit binding (Figure 2).

The binding of [^{125}I]gp120 to fixed CHO/CD4 cells was protein dependent as determined by cell linearity studies. From these studies, we established 100,000 cells per well yielded 80% specific binding (>4,000 total dpm bound). Denaturation of the receptor by exposure to heat (>60°C for 15 minutes) reduced specific binding to approximately 20%. In contrast, heat denaturation totally eliminated specific binding in non-fixed cells.

DISCUSSION

HIV-1 has demonstrated tropism for CD4$^+$ cells *in vitro* and *in vivo* (1-8), however, HIV-1 infectivity of non-CD4$^+$ cells has also been demonstrated *in vitro* (23). A subset of CD4 expressing T lymphocytes, termed helper T cells, were crucial in the differentiation and function of various cellular components of the immune system (1-2). The ability of HIV-1 to directly infect and kill this cell population played a key role in the virus' ability to compromise the cellular immune function (3-8). The time course of helper T cell elimination, however, did not account for the

numerous immune dysfunctions which appeared during the normal course of infection (24). Such extensive disruption of the immune system may arise from HIV-1 ability to alter function of other cells which express the CD4 receptor, e.g. monocytes/macrophages (25-30). The recent identification of CD4 message in cells of non-immune origin, i.e. colorectal endothelial, microglial, and neuroblastoma cells (31-33), implicates the involvement of gp120/CD4 binding in AIDS pathophysiology outside of the immune system.

We report here the characterization of an [^{125}I]gp120 radioligand binding assay designed for high volume screening which utilized a fixed whole cell as a receptor source. We have established that the binding kinetics for this assay were identical to those reported for viable cells. This type of high volume assay enables the identification of gp120 antagonists by comparing the amount of ligand bound to the CD4 receptor in the presence of a single concentration of a test sample. Once an inhibitor has been identified, normally >50% inhibition at 10 μM, the relative potency of that compound is established by determining its inhibitory constant, K_i. Functional bioassays (e.g. syncytium formation) may then be used to determine if that compound is an agonist or antagonist.

In the present study we developed a radioligand binding assay which utilizes [^{125}I]gp120, as the radioligand and paraformaldehyde fixed CHO cells co-transfected with the cDNAs for human CD4 as the receptor source. The use of an iodinated gp120 has the advantage of utilizing a ligand of high specific activity. Specific activities varied from 800 to 1200 Ci/mmol between lots of iodinated

gp120, as calculated by ELISA. At the concentration of approximately 1 nM [^{125}I]gp120 (80,000 dpm) used in concentration-response curves, total binding ranged from 4,000 - 5,000 dpm. We were unable to generate a gp120 ligand of higher specific activity due to the instability of the gp120 molecule.

Initial studies centered around the use of viable CHO/CD4 cells attached to tissue culture plastic. Concentration-response curves resulted in K_is of approximately 10 nM, within the range anticipated from previous studies (14-17). The use of viable cells resulted in significant variability within triplicate well values (approximately 20%) used to generate individual data points in our binding assays. This variability was much greater than values generated following paraformaldehyde fixation. Under routine microscopic examination of cells during each step of the assay, it became apparent that this variability was the result of cell loss. In an attempt to eliminate cell loss, tissue culture plastic was treated with substrates which increase cell attachment, i.e., collagen, poly-l-lysine. Each treatment decreased the number of cells lost during the assay, however, variability was still greater than that seen in fixed cells run under identical conditions.

The need for a protease inhibitior in our assay suggests that either a contaminant proteolytic activity in the recombinant gp120 preparation or some form of endogenous proteolytic activity may be present in our fixed cell preparation. In this assay it is necessary to control for possible proteolytic activity to reduce possible assasy to assay variability.

As another approach to eliminate the variability, we used viable cells in suspension and terminated the assay by centrifugation. Protein analysis of individual assay tubes, following the washes and spins needed to effectively remove unbound [^{125}I]gp120, showed a small but significant variation in protein concentration. Such a variation, along with the time and effort needed to perform a centrifugation assay, made it unacceptable when compared with our [^{125}I]gp120 radioligand binding assay. Based on the statistical information available on high volume screening compiled using the NovaScreen® system, such variability in a high volume format would compromise identification of inhibitory compounds which may comprise <1% of the compounds in libraries tested.

We attempted to eliminate variability by the use of paraformaldehyde fixation. This method of fixation was chosen for several reasons. First, during immunochemistry, CHO/CD4 cells fixed with 2% paraformaldehyde remain attached throughout the rigorous washing steps required in this procedure (Figure 1). Second, the affinity of CD4 for gp120 was encoded for in an immunoglobulin-like domain of its structure suggesting that gp120 binding to the CD4 receptor was similar to antibody/antigen binding. It has also been reported that this fixative is the most promising, causing minimal disruption of protein structure (34). The inability of heat denaturation to totally eliminate specific binding in paraformaldehyde fixed cells suggested that such treatment may actually stabilize the CD4 structure. The ability of both LEU3A and OKT4A to recognize sites associated with gp120 binding after fixation suggested that the CD4 tertiary structure remained intact.

Since protein/peptide binding was similar, in principle, to the binding of an antibody to an antigen, it was thought gp120/CD4 binding may not be significantly altered by the paraformaldehyde fixative. The present study revealed that gp120 concentration-response curves were identical for viable or fixed cell preparations (Figure 3). Both were equally sensitive to variations in the time and temperature of incubation. Furthermore, the inhibitory characteristics of compounds in Table 1 and identified in preliminary screening were identical in fixed versus viable cell assays (data not shown).

A number of compounds reported to inhibit gp120 binding and/or gp120 induced syncytia formation were tested in our fixed cell assay and found to inhibit gp120 binding. Peptide T, previously shown to be an inhibitor of syncytium formation (35), was only a weak displacer of specific [^{125}I]gp120 binding. We were unable to explain the inability of peptide T or its analogue to inhibit gp120/CD4 binding. However, peptide T inhibition of the binding of gp120 binding in the brain may be due to interaction at a receptor pharmacologically distinct from CD4. Several groups have recently reported that HIV-1 is able to infect neuronal cell lines through a non-CD4 mediated pathway (31-33). The inability of the gp120 peptide fragment (254-274) to inhibit [^{125}I]gp120 binding to the CD4 molecule binding corresponds to reports which show this peptide is not associated with the CD4 binding domain of gp120 (36). The inability of this gp120 peptide fragment and the supernatant which remains after immunoprecipitation to inhibit [^{125}I]gp120 binding supports the selectivity of our binding assay.

Saturation studies on fixed cells revealed that [^{125}I]gp120 binds to a single class of high affinity site, K_D= 6 nM, similar to previously reported values for viable cells. In saturation experiments, however, we were unable to reach absolute saturation. A similar phenomenon in viable CD4 expressing cells has been reported by Kozlowski et al. (38), who suggested the inability to saturate may be the result of the inherent stickiness (extensive post-translational glycosylation of gp120). The inherent stickiness of the gp120 molecule was demonstrated in our inability to show specific binding in membrane homogenates of CHO/CD4 cells. We have also noted a similar trend in other radioligand binding assays which utilize proteins or larger peptides as a radioligand, e.g. [^{125}I]TNF (39).

In summary, we have developed and characterized the high affinity binding of gp120 and the CD4 receptor using recombinant forms of both proteins. The use of recombinant gp120 and CD4 provided a margin of safety as compared to the use of active or inactive HIV-1 which allowed us to develop this assay around our high volume screening technology. Further, the use of a CD4 hyper-expressing CHO cell line also reduced the number of cells required per assay and the tissue culture effort required to support a high volume screening effort. The use of paraformaldehyde fixed cells was optimal in this particular radioligand binding assay. Fixation was necessary to eliminate the significant variability in the results obtained using non-fixed cells. This variability was attributed to the loss of cells during the termination of the assay.

This modification did not result in the altering of the binding kinetics of CD4 for gp120. Such an approach is now being examined for its utility in other radioligand binding assays where large proteins serve as the only suitable radioligand source, i.e., growth and differentiation factors.

ACKNOWLEDGEMENTS

We wish to thank Dr. Henrietta Kulaga and Dr. Carl Kaiser for their critical reading of the manuscript and Mrs. Naomi Randolph for her secretarial assistance. This work was supported by NIAID Grant Number U01A127221-02 to Dr. Paul Sweetnam.

Reprint requests should be addressed to Dr. Paul M. Sweetnam, NovaScreen®, a Division of Nova Pharmaceutical Corporation, 6200 Freeport Centre, Baltimore, Maryland 21224-6522.

REFERENCES

1. Reinherz, E.L. and Schlossman, S.F. The differentiation and function of human T lymphocytes. *Cell* 1980;19: 821-7.

2. Rogozinski, L., Bass A., Glickman, E., et al. The T4 surface antigen is involved in the induction of helper function. *J. Immunol.* 1984;132: 735-9.

3. Popovic, M., Sarngadharan, M.C., Read. E. and Gallo, R.C. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-VIII) from patients with AIDS and pre-AIDS. *Science* 1984;224: 497-500.

4. Barre-Sinoussi, F., Chermann, J.C., Rey, F., et al. Isolation of a T-lymphotrophic retrovirus from a patient at risk for the acquired immunodeficiency syndrome (AIDS). *Science* 1983;220: 868-71.

5. Schupbach, J., Popovic, M., Gilden, R.V., Gonda, M.A., Sarngadharan, M.G. and Gallo, R.C. Serological analysis of a subgroup of human T-lymphotrophic retroviruses (HTLV-III) associated with AIDS. *Science* 1984;224: 503-5.

6. Klatzmann, D., Barre-Sinoussi, F., Nugeyre, M.T., et al. Selective tropism of lymphadenopathy associated virus (LAV) for helper-induced T lymphocytes. *Science* 1984;225: 59-63.

7. McDougal, J.S., Mawle, A., Cort, S.P., et al. Cellular tropism of the human retrovirus HTLV-III/LAV: 1. Role of T cell activation and expression of the T4 antigen. *J. Immunol.* 1985;135: 3151-62.

8. Lane, H.C. and Fauci, A.S. Immunologic abnormalities in the acquired immunodeficiency syndrome. *Annu. Rev. Immunol.* 1985;3: 477-500.

9. Lederman, S., Gulick, R., Chess, L. Dextran sulfate and heparin interact with CD4 molecules to inhibit the binding of coat protein gp120 of HIV. *J. Immunol.* 1989;143: 1149-54.

10. Fisher, R.A., Bertonis, J.N., Meier, W., Johnson, V.A., Costopoulas, D.S. and Liu, T. HIV infection is blocked *in vivo* by recombinant soluble CD4. *Nature* 1988;331: 68-76.

11. Hussey, R.E., Richardson, N.E., Kowalski, M., et al. A soluble CD4 protein selectively inhibits HIV replication and syncytium formations. *Nature* 1988;331: 78-81.

12. Smith, D.H., Byrn, R.A., Marsters, S.A., Gregory, T., Groopman, J.E. and Capon, D.J. Blocking of HIV-1 infectivity by a soluble secreted form of the CD4 antigen. *Science* 1987;2:38: 1704-7.

13. Ferkany, J.W. The radioreceptor assay: A simple, sensitive and rapid analytical procedure. *Life Sci.* 1987;41: 881-4.

14. Burt, D.R. Receptor Binding Techniques. Basic Receptor Methods II. Problems of Interpretation In Binding Studies: *Soc. Neurosci. Sup.* 1980: 53-69.

15. Lundin, K., Nygren, A., Arthur, L.O., et al. A specific assay measuring binding of [^{125}I]gp120 from HIV to T4^{+}/CD4^{+} cells. *J. Immuno. Methods* 1987;97: 93-100.

16. Schnitman, S.M., Lane, H.C., Rota, J., Burows, A., Folks, T.M., Kehrl, J.H. Characterization of gp120 binding to CD4 and an assay that measures ability of sera to inhibit this binding. *J. Immunol.* 1988;141: 4181-6.

17. McQuade, T.J., Pitts, T.W., Tarpley, W.G. A rapid solution immunoassay to quantify binding of the human immunodeficiency virus envelope glycoprotein to soluble CD4. *Biochem. Biophys. Res. Commun.* 1989;163: 172-6.

18. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227: 680-2.

19. Smith, D.H. Byrn, R.A., Marsters, S.A., Gregory, T., Groopman, J.E., Capon, D.J. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science* 1987;238: 1704-7.

20. Lasky, L.A., Nakamura, G., Smith, D.H., et al. Delineation of a region of the human immunodeficiency virus Type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* 1987;50: 975-85.

21. Schols, D., Baba, M., Pauwels, R., Desmyler, J., DeClercq, E. Specific interaciton of aurintricarboxylic acid with the human immunodeficiency virus - CD4 cell receptor. *PNAS*. 1989;86: 3322-6.

22. Smith, C.C. Hallberg, P.L. Sacerdote P. Tritiated D-ALA-1 peptide T binding: A pharmacologic basis for the design of drugs which inhibit HIV receptor binding. *Drug Design Res*. 1988;15: 371-80.

23. Chiode, F., Fuerstenberg, S., Gidlund, M. Asjo, B. and Fenyo, E.M. Infection of brain-derived cells with the human immunodeficiency virus. *J. Virol*. 1987;61: 1244

24. Edelman, A.S., and Zolla-Pazner, S. AIDS: A syndrome of immune dysregulation, dysfunction, and deficiency. *FASEB J*. 1989;3:22-30.

25. Jameson, B.A., Rao, P.E., Kong, L.I., et al. Location and chemical synthesis of a binding site for HIV-1 on the CD4 protein. *Science* 1988;240: 1335-8.

26. Koenig, S., Gendelman, H.E., Orenstein, J.M., et al. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* 1986;233: 1089-93.

27. Lucey, D.R., Dorsky, D.I., Nicholson-Weller, A. and Weller P.F. Human eosinophils express CD4 protein and bind human immunodeficiency virus I gp120. *J. Exp. Med*. 1989;169: 327-32.

28. Wahl, L.M., Corcoran, M.L., Pyle, S.W. Arthur, L.O. Harel-Belan, A., Farrar, W.L. Human immunodeficiency virus glycoprotein (gp120) induction of monocyte arachidonic acid metabolites and interleukin 1. *PNAS* 1989;86: 621-5.

29. Ho, D.O. Rota, T.R. Hirsch, M.S. Infection of monocyte/macrophages by human T lymhotropic virus type III. *J. Clin. Invest.* 1984;77: 1712-5.

30. Nicholson, J.K.A., Grass, G.D., Callaway, S.C., McDougal, J.S. *In vitro* infection of human monocytes with human T lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV). *J. Immunol.* 1986;137: 323-9.

31. Ho, D.D., Rota T.R., Schooley, R.T., et al. Isolation of HTLV-III from cerebrospinal fluid and neural tissues of patients with neurologic syndromes related to the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 1985;313: 1493.

32. Harouse, J.M., Kunsch, C., Hartle, H.T., et al. CD4-Independent infection of human neural cells by human immunodeficiency virus Type 1. *J. Virol.* 1989;63: 2527-33.

33. Dewhurst, S., Sakai, K., Bresser, J., Stevenson, M. Evinger-Hodges, M.J. and Volsky, D.J. Persistent productive infection of human glial cells by HIV and by infectious molecular class of HIV. *J. Virol.* 1987;61: 3774.

34. Knigge, K.M. and Piedut, D.T. Immunocytochemistry of receptors using anti-ideotypic antibodies. *Methods Enzymol.* 1990;178: 212-21.

35. Pert, C.B., Hill, J.M., Ruff, M.R., et al. Octapeptides deduced from the neuropeptide receptor-like pattern of antigen T4 in brain potently inhibit human immunodeficiency virus receptor binding and T-cell infectivity. *PNAS.* 1986;83: 9254-8.

36. Lasky, L.A., Nakamura G., Smith, D.H., et al. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* 1987;50: 975-85.

37. Kozlowski, M.R., Hall, E. and Watson, A. Characterization of gp120 binding in CEM cells and hippocampus. *Soc. Neurosci. Abstr.* 1989;15: 273.

38. Kozlowski, M.R., Sandler, P.F. Lin and Watson, A. Brain-derived cells contain a specific binding site for gp120 which is not the CD4 antigen. *Brain Res.* 1991;553: 300-4.

39. Blake, D., Baker, I., Connolly, M., Ferkany, J. and Sweetnam, P. Tumor necrosis factor-alpha (TNF-a-induced) cytotoxicity to NG-108 neuroblastomas. *Soc. Neurosci. Abstr.* 1989;15: 437.