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A RAPID SOLUTION IMMUNOASSAY TO QUANTIFY BINDING OF THE HUMAN IMMUNODEFICIENCY VIRUS ENVELOPE GLYCOPROTEIN TO SOLUBLE CD4

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We developed a particle concentration fluorescent immunoassay to quantify the binding in solution of the human immunodeficiency virus (HIV) external glycoprotein (gp120) to soluble CD4 (sCD4). The assay is rapid (1 hr), quantitative, and requires as little as 0.1 pmole of gp120 per evaluation. We find that gp120, purified from recombinant baculovirus infected insect cells, is suitable for the assay. Moreover, sCD4s obtained either from recombinant E. coli or mammalian cells, consisting of the N-terminal two domains (about 180 amino acids) as well as linked to the active regions of Pseudomonas exotoxin A, bind gp120 similarly.

The first invariant molecule that the human immunodeficiency viruses (HIV-1 and HIV-2) encounter as they begin their assault on cells is the CD4 protein (1). The CD4 molecule is an external glycoprotein of about 435 amino acids (aa); the 370 aa of the CD4 extracellular region is roughly divided into 4 domains which share significant sequence homologies and secondary structural features to the immunoglobulin family. CD4 is found predominantly on the surface of T-helper lymphocytes and cells of the monocyte/macrophage lineage (2). While the physiological role of CD4 on monocytes/macrophages is unknown, on T-cells CD4 is involved in class II major histocompatibilty molecule recognition (2). The evidence is now unequivocal that CD4 is the major cellular receptor for HIV (1). HIV's targeting to cells is by virtue of the strong affinity of its major external glycoprotein (gp120) with CD4 (Kd about 10^{-9}M; Ref. 3); this interaction initiates fusion of HIV and the cell membrane resulting in viral penetration and initiation of infection (1).

Work by others demonstrated that truncated, soluble CD4 molecules (sCD4) obtained from recombinant mammalian or insect cells block the HIV infectivity of cells (3-7). This block presumably results from the competition for HIV by sCD4 and cell-associated CD4. Although initial work utilized sCD4s consisting of the entire four CD4 extracellular domains (370 aa), subsequent work demonstrated that the N-terminal 180 aa (domains 1-2) were sufficient to bind HIV gp120 (8-9). Since HIV gp120:CD4 interaction is required for HIV infection, agents interfering with this association may have utility as anti-HIV therapeutics. We describe herein, a rapid, sensitive immunoassay to quantify sCD4:gp120 binding. We also demonstrate that sCD4s derived from recombinant E. coli after denaturation and refolding bind gp120 indistinguishably compared to mammalian cell derived sCD4.

MATERIALS AND METHODS

HIV gp120 purified from infected H9 cells was obtained from Ellis Reinherz (Dana-Farber Cancer Institute, Harvard; Ref. 10); gp120 was also purified from

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recombinant baculovirus infected insect cells (b-gp120) and purchased from American Biotechnologies Inc., Cambridge, MA. Anti-gp120 (No. 9284) was from Dupont (Wilmington, DE); OKT4-fluorescein isothiocyanate (OKT4-FITC) and OKT4A were from Ortho (Raritan, NJ); Leu-6 and Leu-3A were from Becton Dickinson (Mountain View, CA) and anti-HLA-A,B,C was from Sera-Lab (Westbury, NY). sCD4-183 (a sCD4 consisting of the N-terminal two CD4 domains of 183 aa) and sCD4(178)-PE (a sCD4 containing the CD4 N-terminal 178aa) linked to the 40 KDal active region of *Pseudomonas* exotoxin A; Ref. 11-12) were obtained from recombinant E. coli and provided by Drs. Robert Garlick and Richard Kirschner (Upjohn); sCD4-182 and sCD4-369 (sCD4s containing the N-terminal CD4 182 or 369 aa, respectively, and obtained from recombinant chinese hamster ovary cells) were obtained from Dan Palermo and Dr. Peter Wells (Upjohn). The Fluorescence Concentration Analyzer (FCA) was purchased from Pandex.

The binding of gp120 preparations to sCD4 was quantified by a fluorescent sandwich immunoassay performed in 96-well microtiter dishes equipped in the bottom of the wells with a metricel 0.2 micron membrane (Fig. 1). Generally, 0.025-0.5 pmoles of gp120 was added to 0.1 ml of 1% bovine serum albumin, 10 mM phosphate buffered saline pH 7.4 containing 0.6 pmoles of sCD4-369. After a 15 min incubation at room temperature (rt), approximately 1.5 pmoles of anti-gp120 coupled to Fluoricon polystyrene particles (according to the manufacturer) was added. After an additional 15 min rt incubation 0.6 pmoles of OKT4-FITC was added; the samples were maintained at rt for 15 min and then inserted into the FCA. The protein-antibody conjugates were collected onto the metricel membrane by vacuum filtration, each well was washed once with 0.1 ml of 0.05% NP-40 in PBS and then aspirated to dryness. The fluorescence in each well was read automatically at 535 nm after irradiation of the wells at 485 nm. The above reagent stoichiometry as well as the sequence of reagent additions were determined to give optimal signal/noise ratios; at higher protein concentrations non-linear responses were sometimes obtained.

The gp120 binding of sCD4-183, sCD4-182, and sCD4(178)-PE were evaluated by preincubation with gp120 and then quantifying the blocking effect on sCD4-369 binding. Since OKT4-FITC (the signal antibody) recognizes an epitope on sCD4 near the boundaries of extracellular domains 3-4 (13), this antibody does not recognize sCD4-183, sCD4-182, or sCD4(178)-PE, all of which contain only the N-terminal two sCD4 domains. Binding of gp120 with any of these sCD4s should result, therefore, in inhibition of subsequent gp120:sCD4-369 interaction, resulting in a reduction of the fluorescent signal. For these analyses, 0.4 pmoles of gp120 was preincubated with 0.3, 0.6, 1.2, or 2.4 pmoles of sCD4-183, sCD4-182, or sCD4(178)-PE for 15 min at rt; 0.6 pmoles of sCD4-369 was then added to each well and the assay completed as described above.

RESULTS AND DISCUSSION

We developed a fluorescent sandwich immunoassay to quantify HIV gp120:sCD4 binding in solution (Fig. 1). This assay utilizes two murine monoclonal antibodies which "sandwich" gp120 bound to sCD4-369; one antibody (the capture antibody) recognizes a gp120 epitope previously determined not to interfere with binding to sCD4 (14). The second antibody (OKT4-FITC; signal antibody) is used for detection and recognizes an epitope on sCD4 near the CD4 domain 3-4 boundary (2). We determined that fluorescence was linearly related ($r = 0.99$) to the concentration of HIV gp120 (purified from H9 infected cells) when assayed at 0.025-0.5 pmoles as described in Materials and Methods (Fig. 2; Ref. 10). Due to the potential hazards of routinely preparing gp120 from HIV-infected cells, we were interested in determining whether gp120 obtained from a recombinant source might be suitable

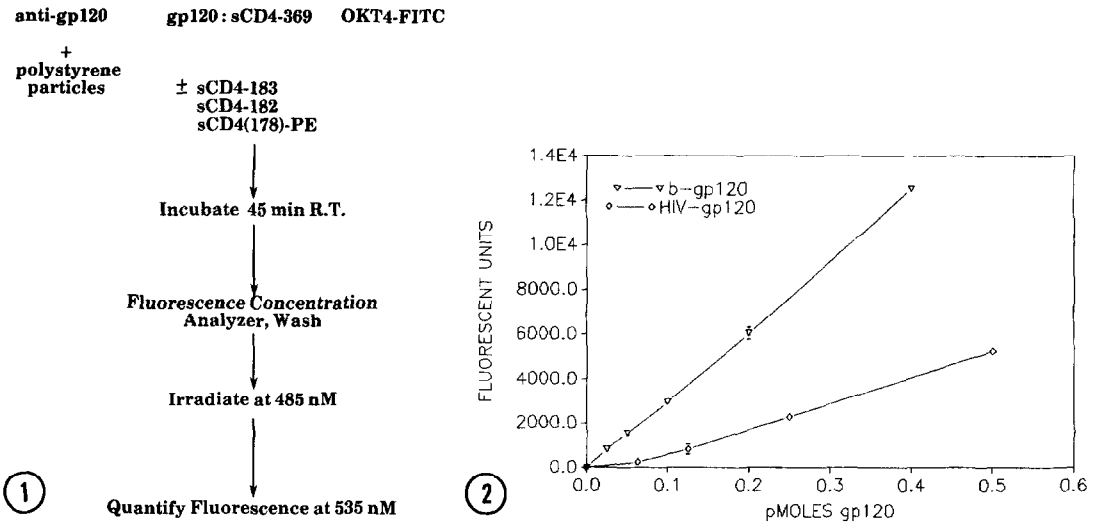

Fig. 1. Overview of gp120:sCD4 fluorescent immunoassay. A fluorescent immunoassay which quantifies the solution binding of HIV gp120 to sCD4s was performed as illustrated and as described in Materials and Methods.

Fig. 2. Quantification of HIV gp120:sCD4 binding. HIV gp120 obtained from infected H9 cells (10) or b-gp120 obtained from recombinant baculovirus infected insect cells were evaluated for binding to sCD4-369 as described in Materials and Methods. The data is presented as the average fluorescent units (with background subtracted) of two samples with bars indicating the data range; symbols without apparent bars had ranges within symbols.

for the assay. We determined that fluorescence was linearly related ($r = 0.99$) to the concentration of b-gp120 (HIV gp120 purified from recombinant baculovirus infected insect cells) from 0.025 to 0.4 pmoles (Fig. 2). Moreover, the fluorescent signal was consistently higher (about 3-5 fold) compared to that obtained with equivalent concentrations of gp120 obtained from infected cells.

Deletion of gp120/b-gp120 or sCD4-369 from the above assay incubations gave only background fluorescence levels (about 2000 units; data not shown); moreover, addition of OKT4A or Leu-3A, monoclonal antibodies known to block gp120:CD4 interaction, at equimolar levels relative to sCD4-369 (0.6 pmoles), also yielded background fluorescence (data not shown). Conversely, addition of the control monoclonal antibodies Leu-6 (anti-CD-1; Ref. 15) or anti-HLA A,B,C (Ref. 16) had little effect on gp120:sCD4-369 interaction (data not shown).

The above data taken together indicates that gp120:sCD4-369 binding is linearly related to gp120 concentration when assayed under the conditions described in Materials and Methods. Moreover, the observed binding is specific as evidenced by OKT4A and Leu-3A blocking, but not Leu-6 or anti-HLA A,B,C interference. Finally, the data indicates that recombinant b-gp120, despite its expected differences in glycosylation compared to mammalian cell derived gp120, is a good substitute for gp120 prepared from HIV infected cells for the routine analysis of sCD4 binding (17).

Data by others indicated that truncated, sCD4 molecules obtained from recombinant mammalian cells, exhibited potent anti-HIV activities (3-7). To determine whether sCD4s containing the N-terminal two CD4 domains bind gp120, we investigated whether preincubating sCD4-183, sCD4-182, or sCD4(178)-PE with gp120 would block subsequent gp120:sCD4-369 interaction (Fig. 3). As described in

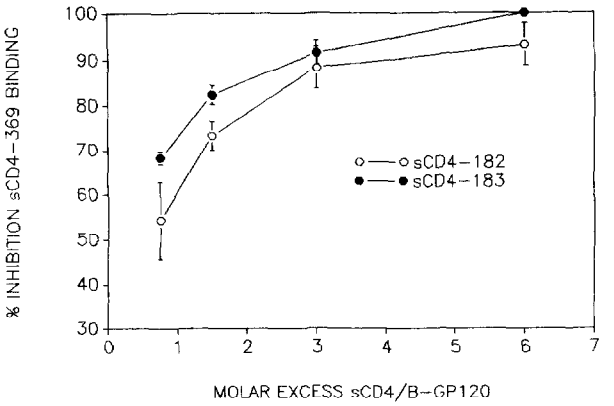

Fig. 3. sCD4-183 derived from recombinant E. coli binds HIV gp120 in a quantitatively indistinguishable way compared to mammalian cell derived sCD4-182. HIV b-gp120 (0.4 pmoles) was preincubated for 15 min at rt with 0.75, 1.5, 3, and 6 molar equivalents of sCD4-183 (from recombinant E. coli) or sCD4-182 (from recombinant mammalian cells). sCD4-369 was added (0.6 pmoles) and the assay performed as described in Materials and Methods. The data is presented as the percentage inhibition of gp120:sCD4-369 binding (control) by preincubation with sCD4-183 or 182. Each data point is the Mean ± S.D., n = 3.

Materials and Methods, since the signal antibody does not recognize two domain sCD4s, we expected that gp120 preincubation with these molecules (if gp120 binding occurred) would prevent subsequent sCD4-369 binding and OKT4-FITC detection.

We determined that preincubation of b-gp120 with 0.75 molar equivalents of sCD4-183, purified from recombinant E. coli after denaturation and refolding, reduced sCD4-369 binding by 68% ± 1 compared to controls. The level of inhibition increased to 92% ± 3 when a 3 molar excess of sCD4-183 was used. Importantly, sCD4-183 blocked gp120:sCD4-369 binding in an indistinguishable way compared to sCD4-182 (Fig. 3); this latter sCD4 is obtained from recombinant mammalian cells and therefore assumed to have proper biological conformation. Finally, we determined that incubation of b-gp120 with 1.5 molar equivalents of sCD4(178)-PE, reduced sCD4-369 binding 60% ± 2. These data strongly suggest that two-domain sCD4s obtained from recombinant E. coli can be refolded to native gp120 binding conformation. This conclusion is supported by the demonstration of the potent anti-HIV activities of sCD4-183 and sCD4(178)-PE (11-12, 18; data not shown).

In summary, we have developed a rapid, sensitive, fluorescent sandwich immunoassay to routinely quantify gp120:sCD4 binding. With this assay we determined that b-gp120 is a good substitute for gp120 derived from HIV infected cells. In addition, we determined that two domain sCD4s obtained from recombinant E. coli or mammalian cells blocked gp120:sCD4-369 binding similarly, suggesting that recombinant E. coli is a good source for sCD4 production. This assay does not require the attachment of either gp120 or sCD4 to plastic (as in the typical ELISA configuration). It, therefore, is a superior way to quantify protein binding, since concerns about conformational changes induced by protein plastic attachment are eliminated. We anticipate that this assay will be useful in the search for specific inhibitors of gp120:sCD4 interaction.

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