Bovine β-lactoglobulin modified by 3-hydroxyphthalic anhydride blocks the CD4 cell receptor for HIV

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Sexual transmission is the most frequent (86%) route of adult HIV-1 transmission worldwide[1]. In the absence of a prophylactic anti-HIV vaccine, other methods of preventing infection should be implemented. Virucidal spermicides have been considered for this purpose, but their application is contraindicated by adverse effects[2]. Anti-HIV drugs[3] or virus-neutralizing monoclonal antibodies[4] are expensive, suggesting that their wide use in topical chemoprophylaxis is unlikely. This emphasizes the importance of developing other methods for preventing HIV transmission. The target cells for sexual and mucosal HIV transmission include T lymphocytes, monocytes/macrophages and dendritic cells[5]. Therefore, compounds blocking HIV-CD4 binding are expected to inhibit virus transmission. In exploring the possibility that chemical modification of food proteins might lead to compounds with anti-HIV-1 activity, we found that bovine β-lactoglobulin (β-LG) modified by 3-hydroxyphthalic anhydride (3HP-β-LG) (1) blocked at nanomolar concentrations the binding to CD4 of human (HIV) and simian (SIV) immunodeficiency virus surface glycoproteins and monoclonal antibodies specific for the HIV binding site on CD4 and (2) inhibited infection by HIV-1, including primary virus isolates, by HIV-2 and by SIV. The inexpensive and widely available source (whey) for production of 3HP-β-LG suggests its potential application (nonparenteral) for diminishing the frequency of HIV transmission.

β-Lactoglobulin (β-LG) modified by 3-hydroxyphthalic anhydride (3HP-β-LG) inhibited the binding between soluble CD4 and the surface (SU) glycoproteins from human immunodeficiency virus, type 1 (HIV-1) strains IIIB and MN, and HIV-2 and from simian immunodeficiency virus (SIV) (Fig. 1*a*). The similarity of results obtained for the distinct SU glycoproteins suggested that the preferential target for 3HP-β-LG was CD4, the common receptor for HIV-1, HIV-2 and SIV (ref. 6). In accordance with this, 3HP-β-LG inhibited the binding to CD4 of monoclonal antibodies Q4120 and OKT4a (Fig. 1*b*; concentration required for 50% inhibition (EC_{50}) = 7 and 12.2 nM, respectively), specific for the HIV SU glycoprotein binding site on CD4 (ref. 7, 8). Binding of the monoclonal antibody OKT4, specific for another domain on CD4 (ref. 8) was not inhibited (EC_{50} > 2.8 μM). Binding of 3HP-β-LG to CD4 was also demonstrated directly (Fig. 2). The association constant (K_a) for CD4-3HP-β-LG binding, as determined from the inhibitory effect of graded quantities of unlabeled 3HP-β-LG on binding to CD4 of biotinyl-3HP-β-LG (ref. 9) was 1.54 ± 0.14 × 10^8 M^{-1}. In comparison, 3HP-β-LG bound to HIV-1_{IIIB} gp120 to a much lesser extent (Fig. 2). Saturating binding to CD4 within 1 minute was accomplished at 3HP-β-LG concentrations ≥140 nM (data not shown).

For 3HP-β-LG to serve as an effective barrier against HIV infection, it ought to bind not only to soluble CD4 but also to cellular CD4. Results shown in Fig. 3 indicate that 3HP-β-LG reacted with $CD4^+$ HeLa cells but not with control HeLa cells. The binding was inhibited by soluble CD4 and was not detected when 3HP-β-LG was replaced by unmodified β-LG on the solid phase. In reciprocal experiments, binding of $CD4^+$ HeLa cells to immobilized anti-CD4 was inhibited by 3HP-β-LG. Pretreatment of cells with 3HP-β-LG was sufficient to accomplish this effect (data not shown).

To explain the remarkable inhibition by 3HP-β-LG of CD4-gp120 binding, a computer-aided docking simulation of CD4-3HP-β-LG association was performed. The CD4 segment S57–N66 encompassing the epitope for monoclonal antibody OKT4a (ref. 8) was docked onto 3HP-β-LG using the strategy of Stoddard and Koshland[10]. The docked position of the peptide was near the region (K69–K99) on 3HP-β-LG (Fig. 4), rich in modified lysines. After superposition of the D1 and D2 domains of CD4 onto the docked peptide followed by unrestrained minimization, several interactions could be discerned in the complex: hydrophobic interaction between F43 of CD4 and modified K83 of 3HP-β-LG; a hydrogen bond between K46 of CD4 and the 3HP portion of modified K47, a salt bridge and hydrogen bond of CD4 R59 with D53 and E74 of 3HP-β-LG, respectively. Residues F43, K46 and R59 represent major contact sites for gp120 binding[11].

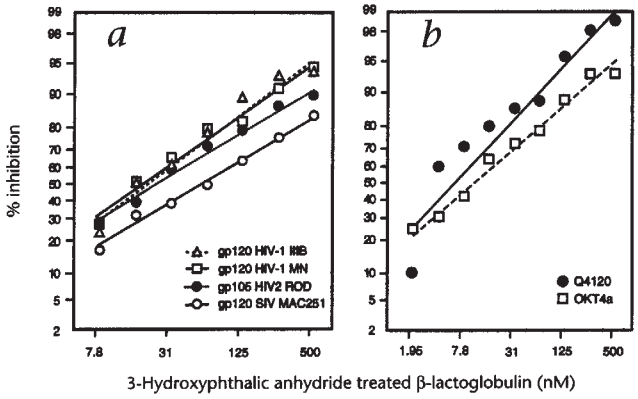

Fig. 1 Inhibitory effect of 3HP-β-LG on the association between recombinant CD4 and distinct CD4-binding proteins. *a*, Binding of horseradish peroxidase (HRP)-labeled CD4 (8.5 pmol added per well) to wells coated with surface envelope glycoproteins of distinct primate immunodeficiency viruses. *b*, Binding to CD4-coated wells of monoclonal antibodies Q4120 and OKT4a (6.3 and 0.63 pmol, respectively, added per well). Similar results were obtained with CD4 captured onto polystyrene by monoclonal antibody OKT4 (data not shown). Absorbance readings at 450 nm (A_{450}) corresponding to binding reactions *a, b* in the absence of 3HP-β-LG were in the range of 0.58 to 1.63. The percentages of inhibition for each 3HP-β-LG concentration were calculated and plotted after logit transformation against log (3HP-β-LG (nanomolar)). The corresponding linear regressions (correlation coefficients (*r*) were 0.97 to 0.996) are presented.

The compound 3HP-β-LG inhibited infection of continuous T-cell lines by laboratory HIV-1 strains, by HIV-2, SIV and a chimeric HIV-1/SIV virus (Table 1). 3HP-β-LG also blocked infection of T-cell lines and peripheral blood mononuclear cells (PBMCs) by HIV-1 from a panel of zidovudine (AZT)-resistant viruses and clinical isolates, respectively (Table 1). 3HP-β-LG inhibited fusion between uninfected cells and cells infected with HIV-1_{IIIB} or HIV-2 (EC_{50} = 96 ± 0.3 and 163 ± 17 nM, EC_{90} = 145 ± 18 and 216 ± 30 nM, respectively). Data concerning the *in vitro* cytotoxicity of 3HP-β-LG for uninfected cells are shown in Table 1. The selectivity index (SI = concentration at which 50% of uninfected cells became unviable (IC_{50}) after six days' incubation with 3HP-β-LG divided by EC_{50}) for the distinct virus-cell systems was 36 to 3,941. The SI corresponding to a two-hour exposure of cells to 3HP-β-LG was ≥367 to ≥36,800, calculated from $IC_{50} \geq 81$ μM for all cell types listed in Table 1.

Cell-to-cell HIV transmission, rather than infection by cell-free HIV, represents a more efficient mode for initiating infection *in vitro*[12]. Therefore, expression of β-galactosidase (β-gal) in HeLa-CD4-LTR-β-gal cells[13] was measured in the absence or presence of graded levels of 3HP-β-LG under conditions in which both cell-free and cell-to-cell virus infection may occur simultaneously. 3HP-β-LG inhibited infection of these cells by both HIV-1 and HIV-2 infected cells (albeit at concentrations higher than those required for inhibition of infection with cell-free virus (Table 1)), as indicated by decreased production of the reporter gene product, β-gal (Table 2). This suggests that 3HP-β-LG may prevent infection by biological fluids containing both cell-free and cell-associated HIV.

Unlike AIDS therapeutics targeted to the virus that frequently lead to drug resistance, agents targeted to the CD4 cell receptor for HIV have the potential to inhibit a wide range of HIV-1 isolates and are much less likely to elicit the emergence of drug-resistant mutant viruses. A recombinant $CD4IgG_2$ heterotetramer (unlike soluble CD4)[14], as a competitor with cellular CD4, or anti-CD4 antibodies[15] are expected to prevent virus transmission notwithstanding subtype variability. This report provides evidence that β-LG, the most abundant globular protein of milk and the major protein component of whey (11–22 μM)[16], can be converted into a compound (3HP-β-LG) that blocks the binding site on CD4 for HIV and SIV, and functions as a surrogate anti-CD4 antibody.

Considering its antiviral potency, ease of preparation and practically unlimited and inexpensive source (the worldwide production of whey is ~86 billion kg annually[16]) and the widely repeated exposure of humans to untreated β-LG in the form of consumed dairy products, the application of 3HP-β-LG to AIDS prophylaxis appears economically more viable as compared with recombinant proteins and anti-HIV or anti-CD4 monoclonal antibodies, respectively. Unlike virus neutralizing or virucidal compounds which have to inactivate HIV-1 instantaneously to be effective in preventing sexual transmission, 3HP-β-LG would be preapplied topically to generate a barrier against infection. 3HP-β-LG at concentrations preventing HIV-1 infection *in vitro* is in large excess over CD4 cell receptors and is also expected to block most if not all these receptors within minutes under *in vivo* conditions using vehicles with adequate 3HP-β-LG bioavailability. CD4 receptors on cells pretreated *in vitro* with 3HP-β-LG and subsequently washed were not accessible to anti-CD4 monoclonal antibodies. This suggests that after removal of the topically applied 3HP-β-LG formulation, CD4 receptors would remain blocked. Combinations of CD4 blocking and virucidal compounds would be expected to result in the most effective approach to prevent HIV-1 sexual transmission.

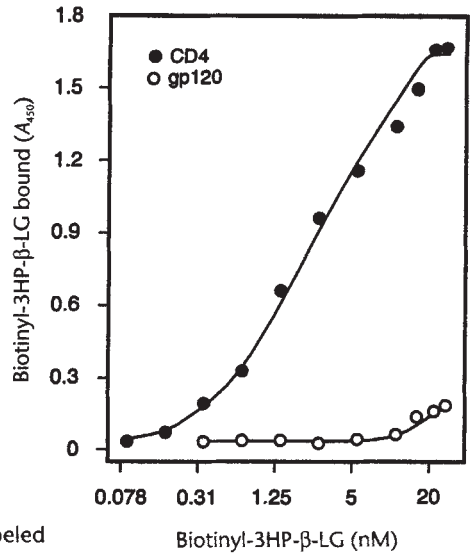

Fig. 2 Preferential binding of 3HP-β-LG to CD4 as compared with HIV-1_{IIIB} gp120. Graded quantities of biotinylated 3HP-β-LG were added to gp120- and to CD4-coated wells, respectively. After overnight incubation at 25 °C, the wells were washed and bound biotinyl-3HP-β-LG was determined from subsequent binding to the wells of HRP-labeled streptavidin. Absorbance (A_{450}) values corresponding to biotinyl-3HP-β-LG bound to gelatin-coated control wells (range 0–0.034) were subtracted from values corresponding to gp120- or CD4-bound 3HP-β-LG and the differences were plotted. Unlabeled 3HP-β-LG (556 nM) suppressed the binding of biotinyl-3HP-β-LG (111 nM) to CD4 by 94%. Biotinylated untreated β-LG bound neither to gp120 nor to CD4 ($A_{450} \leq 0.040$). Similar results were obtained using unlabeled 3HP-β-LG, the binding of which to the wells was detected by anti-β-LG antibodies, which recognized untreated and 3HP-treated β-LG equally, suggesting that the antigenicity of β-LG was not measurably altered by the chemical modification (data not shown).

The results of *in vitro* assays indicating the potent anti-HIV activity of 3HP-β-LG justify further preclinical development in various formulations suitable for topical applications that would ensure adequate bioavailability, *in vivo* retention time and penetration onto relevant tissues.

Methods

Reagents. The reagents used were β-lactoglobulin from bovine

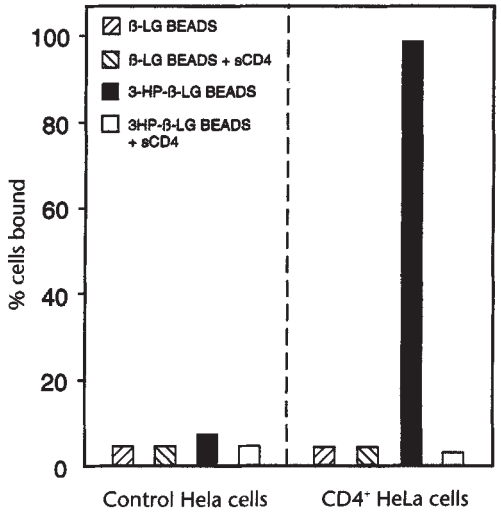

Fig. 3 Binding of $CD4^+$ and control HeLa cells, respectively, to magnetic beads coated with β-LG or with 3-HP-β-LG and binding inhibition by soluble CD4 (420 pmol in 1 ml). Bound and unbound cells were quantitated as described[20].

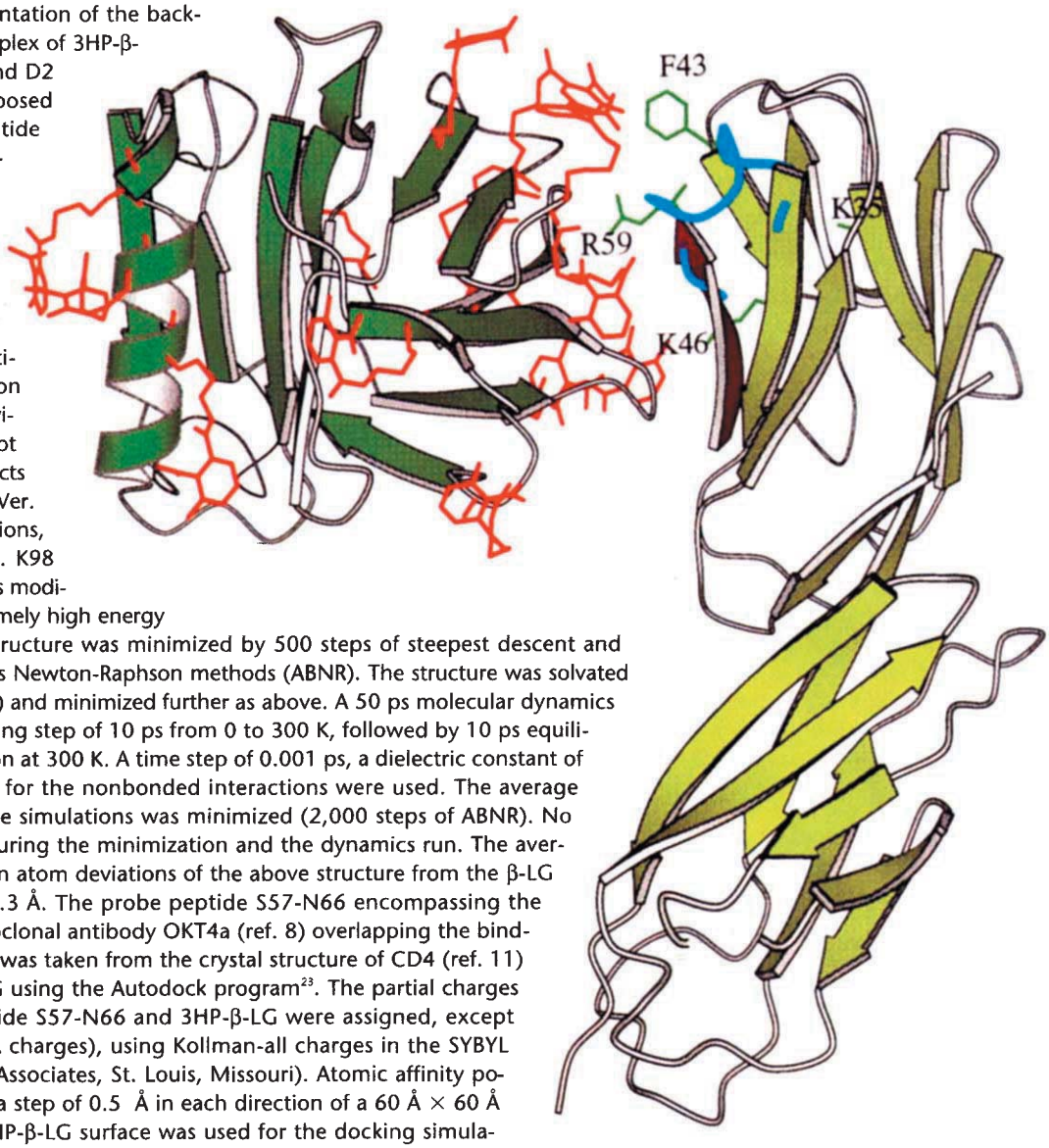

Fig. 4 Molscript[21] representation of the backbone structures of the complex of 3HP-β-LG (green) and CD4 D1 and D2 domains (yellow) superimposed on the docked CD4 peptide S57-N66 (blue). Red-colored side chains indicate the modified lysines on 3HP-β-LG. The green-colored side chains indicate contact sites on CD4 for gp120 (ref. 11). The monoclonal antibody OKT4a binding site on CD4 (ref. 8) is indicated in violet. β-LG lysines (except K98) were modified to adducts with 3-HP using QUANTA (Ver. 4.0, Molecular Simulations, Burlington, Massachusetts). K98 is buried within β-LG, and its modification generated an extremely high energy structure. The 3HP-β-LG structure was minimized by 500 steps of steepest descent and 2000 steps of adopted basis Newton-Raphson methods (ABNR). The structure was solvated with water molecules (10 Å) and minimized further as above. A 50 ps molecular dynamics (MD) was run using a heating step of 10 ps from 0 to 300 K, followed by 10 ps equilibration and 30 ps simulation at 300 K. A time step of 0.001 ps, a dielectric constant of 1 and a 15 Å cut-off value for the nonbonded interactions were used. The average structure resulting from the simulations was minimized (2,000 steps of ABNR). No constraints were applied during the minimization and the dynamics run. The average C-atom and main chain atom deviations of the above structure from the β-LG crystal structure[22] were ~1.3 Å. The probe peptide S57-N66 encompassing the epitope for anti-CD4 monoclonal antibody OKT4a (ref. 8) overlapping the binding site for gp120 (ref. 11) was taken from the crystal structure of CD4 (ref. 11) and docked onto 3HP-β-LG using the Autodock program[23]. The partial charges for each atom in the peptide S57-N66 and 3HP-β-LG were assigned, except the 3HP portion (QUANTA charges), using Kollman-all charges in the SYBYL program (Ver. 6.1, Tripos Associates, St. Louis, Missouri). Atomic affinity potentials were calculated in a step of 0.5 Å in each direction of a 60 Å × 60 Å × 60 Å grid. The entire 3HP-β-LG surface was used for the docking simulations. No conformational flexibility was allowed to either 3HP-β-LG or the peptide S57-N66 during docking. One hundred individual docking simulations were performed using a schedule of 50 cycles, each composed of 3,000 steps accepted or rejected, starting with a high temperature (k_BT = 50 kcal/mol) and a decrement of 0.95/cycle. Three individual runs were performed placing the S57-N66 peptide at different locations on the surface of 3HP-β-LG. All these simulations docked the peptide at the same position.

whey (β-LG; 3× crystallized; Sigma); 3-hydroxyphthalic anhydride (3HP; Aldrich, Milwaukee, Wisconsin); CD4 (Genentech, South San Francisco, California); HIV-1_{IIIB} gp120, HIV-2 gp105, SIV_{mac251} gp120 and horseradish peroxidase (HRP)-labeled CD4 (all from Intracel, Cambridge, Massachusetts); HIV-1 MN gp120 (Agmed, Bedford, Massachusetts); anti-CD4 mAbs OKT4, OKT4a (Ortho Diagnostics, Raritan, New Jersey) and Q4120 (Sigma); antisera against bovine whey proteins (Accurate Chemical and Scientific Corporation, Westbury, New York); normal mouse IgG isotypes (Cappel-Organon Teknika, Durham, North Carolina); HRP-labeled streptavidin (Amersham, Arlington Heights, Illinois) and HRP-labeled second antibodies against mouse, rabbit and sheep IgG (Sigma).

Chemical modification of β-LG. β-LG (111.1 or 11.1 μM in 0.1 M phosphate pH 8.5) was treated with 3HP (1.19 M in dimethylformamide), added in five aliquots (final concentration, 60 mM) in 12-min intervals, and the pH was maintained at 8.5. The mixture (10 ml) was kept for another 1 h at 25 °C, dialyzed against 4 l of 0.15 M NaCl, 10 mM phosphate, pH 7.4 (PBS) overnight, redialyzed for 4 h with fresh buffer, and filtered through 0.45-μm syringe filters (Acrodisc; Gelman Sciences, Ann Arbor, Michigan). Treatment with 2,4,6-trinitrobenzene-sulfonic acid (TNBS) (28.4 mM) was carried out at pH 9.0 in phosphate buffer for 3 h at 25 °C.

Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, Illinois). To quantitate lysine in the original and the chemically modified preparations, they were treated with TNBS and dialyzed against 0.1 M $NaHSO_3$. The absorbance at 420 nm (A_{420}) of the dialyzed preparations and their appropriate dilutions in 0.1 M $NaHSO_3$ were measured. Quantitation of lysine,

determined from calibration curves relating A_{420} values to lysine concentrations in standards, revealed that 11 to 14 of the 15 β-LG lysines were modified, depending on the 3HP/β-LG ratios. The preparations had similar properties.

Measurement of anti-HIV activity. Cells (10^4; ~300/mm^2; Table 1) were infected at a multiplicity of infection (MOI) = 0.0045 with distinct HIV-1 strains (isolates), HIV-2-ROD, a chimeric HIV-1/SIV_{mac} (SHIV-4), and SIV_{mac}, respectively, in the absence or presence of 3HP-β-LG (0.87 to 889 nM, corresponding to an estimated ~2×10^2 to ~2×10^5-fold molar excess of 3HP-β-LG over cellular CD4). Infection by primary HIV-1 isolates from plasma of infected individuals was determined using peripheral blood mononuclear cells (PBMCs)[17] (1.5×10^6) from HIV-1-negative donors. Cytopathic effects (CPE), nucleocapsid antigen production, and cell fusion using HIV-1_{IIIB}-infected H9 cells and HIV-2_{ROD}-infected U937 cells were measured as described[18]. 3HP-β-LG cytotoxicity for uninfected cells was measured by trypan blue dye exclusion. All assays were performed in triplicate.

The inhibitory effect of 3HP-β-LG on induction of β-gal in cells containing an integrated β-gal gene that is under the control of a truncated HIV-1 long terminal repeat (LTR), cocultivated with infected cells was also measured. HeLa-CD4-LTR-β-gal cells[13] (2×10^5 in 100 µl) in 96-well flat-bottomed Corning tissue culture plates were mixed with 50 µl of 0 to 13.5 µM 3HP-β-LG. After 1 h at 25 °C, H9 and U937 cells chronically infected with HIV-1_{IIIB} and HIV-2_{ROD}, respectively (5×10^4 cells in 50 µl; prewashed to remove cell-free virus) were added. The cells were suspended in RPMI 1640 medium with 10% FBS. After 48 h at 37 °C, the cells were lysed with 50 µl of 5% Triton X-100 containing protease inhibitors (phenylmethyl-sulfonyl fluoride, leupeptin and pepstatin all at 10 µg/ml). β-gal protein in 1:10 diluted lysates was quantitated using an ELISA kit from 5 Prime→3 Prime Inc. (Boulder, Colorado).

Table 1 Antiviral activity of 3-hydroxyphthalic anhydride treated bovine β-lactoglobulin (3HP-β-LG) against primate immunodeficiency viruses

Virus	Cells (IC_{50} ± s.d. (nM))	EC_{50} ± s.d. (nM) as measured by inhibition of		EC_{90} ± s.d. (nM) as measured by inhibition of		IC_{90} ± s.d. (nM)
		nucleocapsid antigen production	CPE	nucleocapsid antigen production	CPE	
A. Laboratory HIV-1 strains						
IIIB		3.3 ± 0.2	6.7 ± 1.2	5.6 ± 0.3	11.5 ± 3.2	
MN		31.1 ± 5.6	15.8 ± 2.9	132 ± 49	43 ± 15	
RF		2.2 ± 0.3	11.4 ± 3.8	3.8 ± 0.6	30 ± 12	
SF2		16.7 ± 1.3	26.1 ± 2.6	39 ± 8	60 ± 15	
V_{32}		23.3 ± 6.5	24.1 ± 3.2	49 ± 20	45 ± 5	
B. AZT-resistant HIV-1 panel	MT-2 (8,670 ± 1,220)					15,400 ± 1,900
Pre-drug isolate 629		114 ± 18	83 ± 4	203 ± 14	195 ± 6	
Post-drug isolate 1075, Intermediate resistant		100 ± 11	100 ± 17	211 ± 11	196 ± 12	
Post-drug isolate 629, resistant		80 ± 7	129 ± 5	163 ± 50	211 ± 9	
C. Primary HIV-1 isolates						
301593	PBMC (7,000 ± 280)	135 ± 2	–*	220 ± 9	–	14,200 ± 330
301660		75 ± 10	–	186 ± 102	–	
302054		195 ± 52	–	554 ± 202	–	
302056		105 ± 7	–	606 ± 49	–	
D. Chimeric HIV-1/SIV (SHIV-4)	MT-2 8,670 ± 1,220)	3.1 ± 0.2	7.5 ± 1.5	8.3 ± 2.1	13.2 ± 2.6	15,400 ± 1,900
E. HIV-2_{ROD}		46 ± 4	221 ± 19	129 ± 5	793 ± 132	
F. $SIV_{mac}251$	CEM-174 (9,220 ± 780)	63 ± 13	–	114 ± 40	–	18,600 ± 4,900

Abbreviations: $IC_{50(90)}$, concentrations at which 50% and 90% of uninfected cells became unviable after six days of incubation with 3HP-β-LG; $EC_{50(90)}$, concentrations for 50% and 90% inhibition of cytopathic effect (CPE) and nucleocapsid antigen production, respectively, which were calculated using a computer program kindly provided by T.-C. Chou, Memorial Sloan-Kettering Cancer Center, New York.
*CPE not measurable by spectrophotometry where indicated by a hyphen.

Table 2 Inhibition by 3-hydroxyphthalic anhydride-treated bovine β-lactoglobulin (3-HP-β-LG) of β-galactosidase induction in HeLa-CD4-LTR-β-gal cells[13] cocultivated with HIV-1- and HIV-2-infected cells, respectively

Cell line used for infection	EC_{50} ± s.d. (nM)	EC_{90} ± s.d. (nM)
H9-HIV-I_{IIIB}	75 ± 14	590 ± 107
U937-HIV-2_{ROD}	118 ± 24	2,786 ± 561

HeLa-CD4-LTR-β-gal cells produced ~0.5 to 1 ng/ml of β-gal in the absence of infected cells.
Abbreviations: $EC_{50(90)}$, concentrations at which the production of β-gal was inhibited by 50% and 90%, respectively, as compared with control cultures to which 3HP-β-LG was not added (= 22.4 and 11.6 ng/ml of β-gal for cocultures containing HIV-1- and HIV-2-infected cells, respectively).

Enzyme-linked immunosorbent assays (ELISA). ELISA was carried out as described[19] using wells coated with CD4 or envelope glycoproteins from distinct primate immunodeficiency viruses (1.7 and 0.84 pmol, respectively, added per well). Reagents binding to the respective proteins (mAbs and antisera) were added to the wells overnight at 25 °C. The bound reagents were detected by HRP-labeled probes (second antibodies in 0.14 M NaCl, 0.01 M Tris, 0.01% sodium merthiolate, pH 7.2 (TS) containing 0.1% Tween 20 and 10% serum of the same species as were the second antibodies, or streptavidin in TS containing 0.25% gelatin) diluted 1:1000 from commercial stock solutions. After incubation for 2 h at 37 °C, the wells were washed with TS, and bound HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Maryland). ELISA kits for HIV-1 and SIV nucleocapsid antigens, respectively, were from Coulter Immunology, Hialeah, Florida.

Binding of cells to magnetic beads coated with β-LG and 3HP-β-LG, respectively. β-LG and 3HP-β-LG (56 μM), respectively, were reduced in 10 mM phosphate, 1 mM EDTA, pH 5.0 containing 1 mM Tris(2-carboxyethyl)phosphine (Pierce) for 45 min at 25 °C, followed by pH adjustment to 7.5 by 1 *N* NaOH. Subsequently, *N*-iodoacetyl-N'-biotinyl hexylenediamine (Pierce) was added to 1.96 mM. After 2 h at 25 °C, the biotinylated proteins were dialyzed against PBS.

Magnetic beads (5 mg in 1 ml PBS containing 1 mg/ml BSA (PBS-BSA); BioMag streptavidin; PerSeptive BioResearch Products, Cambridge, Massachusetts) were mixed with 111 nmol of biotinylated β-LG and 3HP-β-LG, respectively, overnight at 25 °C. The beads were washed 10 times with PBS-BSA and stored at 4 °C (concentration 5 mg/ml). Binding of cells (5×10^5) to 3HP-β-LG or anti-CD4 (PerSeptive BioResearch) magnetic beads (250 μg) was measured[20] using chicken serum (10%) in the diluent. Binding of $CD4^+$ HeLa cells to anti-CD4 beads was measured in the absence and presence of 3HP-β-LG (278 pmol) or using 3HP-β-LG pretreated cells washed with PBS-BSA (1 ml per 10^6 cells).

Acknowledgments

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