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3-Hydroxyphthaloyl-β-lactoglobulin. II. Anti-human immunodeficiency virus type 1 activity in *in vitro* environments relevant to prevention of sexual transmission of the virus

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Summary

It is anticipated that the rate of sexual transmission of viruses could be substantially decreased by the use of topical chemical barrier methods. Chemical modification of bovine β-lactoglobulin (β-LG), the major protein of whey, led to the generation of a potent inhibitor (designated 3HP-β-LG) of human immunodeficiency virus type 1 (HIV-1) infection which was also active against herpesviruses. Compounds intended for topical application to prevent sexual transmission of viruses need to maintain their antiviral activity at pH <<7, corresponding to an acidic vaginal environment, and in the presence of seminal fluid. Results presented here show that the binding of 3HP-β-LG to the CD4 receptor for HIV, involved in the anti-HIV-1 activity of this compound, decreases with decreasing pH. The presence of seminal fluid also decreased the binding of 3HP-β-LG to CD4 and diminished the inhibitory effect of the compound on CD4–gp120 binding. 3HP-β-LG was shown to bind Zn^{++}, and the inhibitory effect of seminal fluid could be substantially diminished by chelating Zn^{++} with ethylenediaminetetraacetate. Saliva had no effect on 3HP-β-LG binding to CD4 or on its interference with gp120–CD4 binding. The decreased 3HP-β-LG–CD4 binding and the concomitant reduction of gp120–CD4 binding inhibition by 3HP-β-LG at low pH and in the presence of seminal fluid could be compensated for by an increase of the 3HP-β-LG concentration and by adding Zn^{++} chelators to 3HP-β-LG. These results provide a background for the design of 3HP-β-LG formulations for topical use.

Keywords: Bovine β-lactoglobulin; 3-hydroxyphthalic anhydride; HIV; CD4; seminal fluid.

Introduction

Bovine β-lactoglobulin (β-LG) chemically modified by treatment with 3-hydroxyphthalic anhydride (3HP) inhibits infection by laboratory strains and clinical isolates of HIV-1, by HIV-2 (Neurath *et al.*, 1996a) and by herpes simplex virus types 1 and 2 (Neurath *et al.*, 1996b). The modified protein, designated 3HP-β-LG, is easy to prepare inexpensively on a large scale from sources that can be pasteurized without affecting the antiviral activities of the final product. This contributes to its safe use for potential topical application to prevent sexual transmission of HIV-1 and other viruses (Neurath *et al.*, 1997). However, such application is possible only if the antiviral activities are maintained in *in vitro* systems that mimic the environment within sites intended for topical application. Thus, it would be expected that a compound intended for prophylactic application would have antiviral activity at around pH 4, occurring in the acidic vaginal environment of healthy women (Voeller & Anderson, 1992; Alexander, 1996), and that its activity would be preserved in the presence of seminal fluid (SF). The unique feature of the anti-HIV-1 activity of 3HP-β-LG is that it involves protein–protein interactions (Neurath *et al.*, 1996a,b) which could be influenced by the pH of the environment and possibly by the presence of other proteins. Results presented here show that the inhibitory activity of 3HP-β-LG is indeed affected by pH and by components of human SF and that the resulting problems can be overcome by appropriate adjustments in the concentration and formulation of 3HP-β-LG.

Materials and Experimental Procedures

Reagents

Most reagents used are described in the preceding paper in this journal (Neurath *et al.*, 1997). The following additional reagents were used: anti-CD4 MAbs OKT4a

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(Ortho Diagnostics, Raritan, N.J., USA) and Q4120 (Sigma); magnetic beads with linked anti-CD4 MAbs specific for the gp120 binding site on CD4 (PerSeptive BioResearch Products, Cambridge, Mass., USA); HeLa $CD4^+$ cells, obtained from R Axel (Columbia University, New York, N.Y., USA; Maddon *et al.*, 1986); human SF (New England Immunology Associates, Cambridge, Mass., USA); murine anti-CD4 MAbs and horseradish peroxidase (HRP)-labelled polyclonal rabbit anti-CD4 antibodies (Agmed, Bedford, Mass., USA); SulfoLink coupling gel (Pierce). Saliva was collected from volunteers; sodium merthiolate was added to a final concentration of 200 μg mL^{-1} to prevent bacterial contamination. 3HP-β-LG was prepared by chemical modification of bovine β-LG and biotinylated as described (Neurath *et al.*, 1997).

ELISA

ELISA was carried out as described (Neurath *et al.*, 1992a, 1997). To quantify CD4 in SF, serial twofold dilutions of SF in 0.14 M NaCl, 0.01 M Tris–HCl pH 7.2 (TS) containing 10 mg mL^{-1} bovine serum albumin (BSA) were added to wells coated with 100 ng of anti-CD4 MAb (Agmed) and post-coated with BSA. The pH of SF was adjusted by addition of CH_3COOH/CH_3COONa buffers to a final concentration of 0.1 M. After overnight incubation at 25°C, the wells were washed with TS containing 0.01% sodium merthiolate. Subsequently, the wells were incubated with 100 μL of HRP-labelled rabbit anti-CD4 in TS containing 10 mg mL^{-1} BSA and 1% normal rabbit serum for 2 h at 37°C. The wells were washed as above and bound HRP was quantified as described (Neurath *et al.*, 1997). The amount of CD4 in the samples was determined from calibration curves relating absorbance readings to concentrations of purified recombinant CD4.

Binding of HeLa $CD4^+$ cells to magnetic beads coated with 3HP-β-LG and anti-CD4 MAbs

3HP-β-LG was biotinylated and subsequently bound to streptavidin-coated magnetic beads as described (Neurath *et al.*, 1996a). Binding of $CD4^+$ HeLa cells to the 3HP-β-LG beads and to anti-CD4 beads was measured in the absence and presence of unlabelled 3HP-β-LG as described (Neurath *et al.*, 1992b, 1996a).

Affinity chromatography of SF proteins on immobilized 3HP-β-LG

One mL of SF, to which EDTA (final concentration 25 mM) was added, was applied to a column of 3HP-β-LG linked to a SulfoLink gel (7 mg of 3HP-β-LG per ml of gel) prewashed with TS–25 mM EDTA. After applying SF, the column was washed with TS–25 mM EDTA until the absorbance of the eluate approached 0. Subsequently, the adsorbed proteins were eluted with 0.05 M $(CH_3-CH_2)_2NH$ pH 11.5. The pH of the fractions containing the eluted proteins was adjusted with 1 M CH_3COOH to neutrality. The column used for chromatography was prepared by covalent linking of 3HP-β-LG reduced with Tris (2-carboxyethyl)-phosphine (Neurath *et al.*, 1996a) to the SulfoLink gel.

Measurement of anti-HIV-1 activity

MT-2 cells (10^4) were infected at a m.o.i. of 0.0045 with $HIV\text{-}1_{IIIB}$ in the presence or absence of 3HP-β-LG. CPE and p24 nucleocapsid antigen production were measured as described (Jiang *et al.*, 1993). To measure the anti-HIV-1 activity of 3HP-β-LG, the pH of culture medium (RPMI-1640 containing 10% foetal bovine serum and 1% penicillin and streptomycin) was adjusted to 4.0 to 7.0 in 0.5 pH unit increments by adding 1 M HCl. 3HP-β-LG, at different concentrations diluted in the pH-adjusted culture media (25 μL), was mixed with 25 μL of MT-2 cells (4×10^5 mL^{-1}) and 20 min later with $HIV\text{-}1_{IIIB}$ (m.o.i. 0.0045), both suspended in the corresponding culture media with the same pH. After incubation at 37°C for 30 min, 125 μL of regular culture medium (pH 7.2) was added. After 1 h, half of the culture supernatant was replaced by regular culture medium. p24 antigen in the culture supernatants and CPE were detected on days 4 and 6, respectively, as described (Jiang *et al.*, 1993).

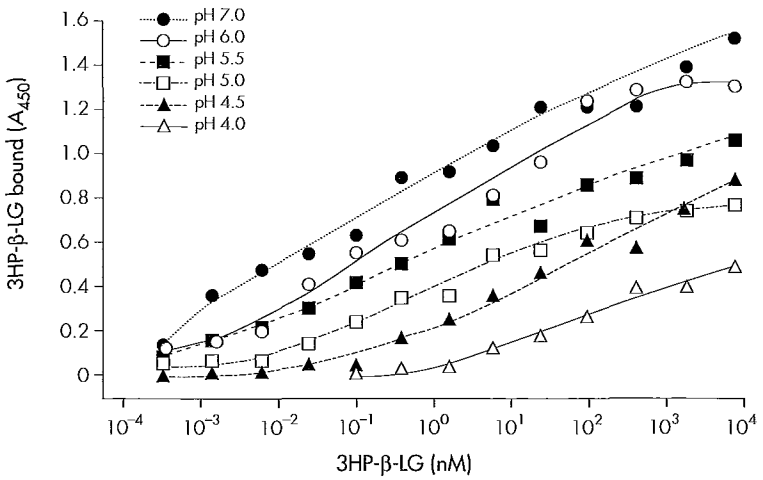
Results

pH dependence of 3HP-β-LG binding to CD4 and of its inhibitory effect on HIV-1 gp120 binding to CD4

Results of earlier studies (Neurath *et al.*, 1996a, 1997) indicated that 3HP-β-LG binds with relatively high affinity to the CD4 receptor for HIV-1 and suggested that this binding contributes to its anti-HIV-1 activity. Owing to understanding of the protein–protein interactions involved in the anti-HIV-1 activity of 3HP-β-LG, it is possible to estimate the effect of pH on antiviral activity by studying the pH dependence of 3HP-β-LG–CD4 binding and its effect on subsequent CD4–gp120 association.

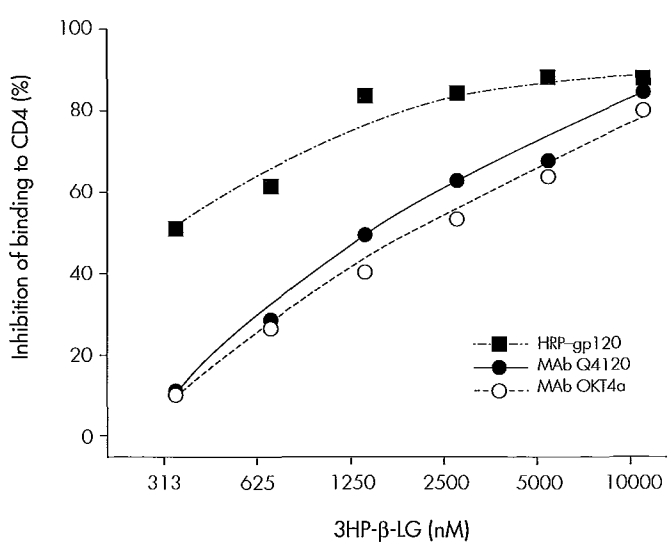
Initial experiments in which the binding of biotinylated 3HP-β-LG to CD4 was measured indicated that a pH decrease from 7.2 to 4.0 resulted in diminished 3HP-β-LG–CD4 association (data not shown). Since the physiological pH at the site of intended topical application of the compound can vary between pH ≤4 to pH 7 or higher (Voeller & Anderson, 1992), the binding of unlabelled 3HP-β-LG to CD4 at wider pH and concentration ranges was studied, quantifying the bound 3HP-β-LG from subsequent binding of anti-3HP-β-LG antibodies to the CD4–3HP-β-LG complexes. A decrease in pH resulted in diminished 3HP-β-LG binding to CD4 throughout the entire pH range tested (Fig. 1).

Figure 1. Binding of 3HP-β-LG to CD4 at different pH.



3HP-β-LG at graded concentrations in 0.1 M CH_3COOH/CH_3COONa buffers of different pH containing 1% BSA and 0.25% gelatin were added to CD4-coated wells overnight at 25°C. The wells were washed and bound 3HP-β-LG was subsequently detected by ELISA using 1:1000 diluted rabbit antiserum against 3HP-β-LG, followed by HRP-labelled anti-rabbit IgG; 0.14 M NaCl, 20 mM Tris-acetate was used for pH 7 and 6 buffers and 0.14 M NaCl, 20 mM acetic acid/sodium acetate buffers were used for pH 4 to 5.5. Absorbance readings corresponding to control wells to which 3HP-β-LG was not added or to wells coated with BSA and gelatin, instead of CD4, to which 3HP-β-LG was added, were both 0.

Figure 2. Inhibitory effect of 3HP-β-LG at pH 4.0 on binding to CD4 of HRP-labelled gp120 and of anti-CD4 MAbs Q4120 and OKT4a.

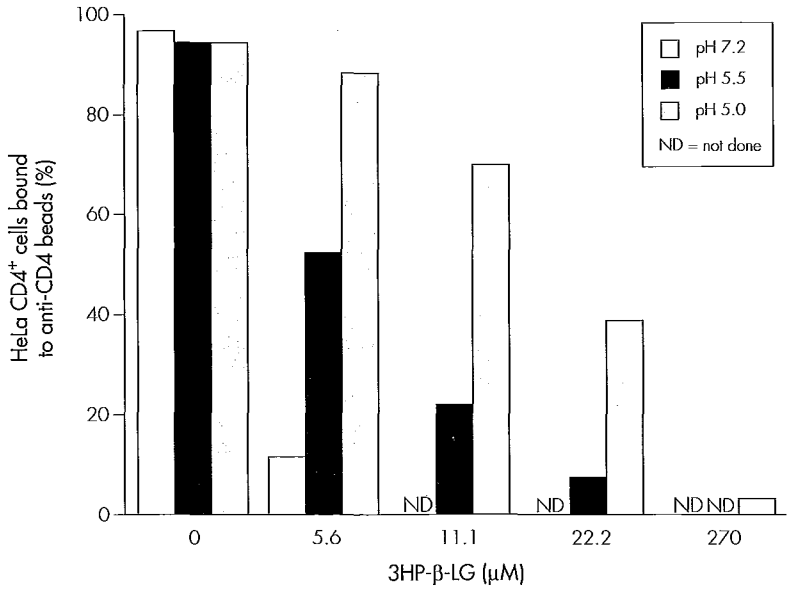


Graded quantities of 3HP-β-LG in 0.1 M CH_3COOH/CH_3COONa pH 4.0 containing 1% BSA and 0.25% gelatin were added to CD4-coated wells. After 2 h at 25°C, the wells were washed and HRP–gp120 (200 ng $well^{-1}$) and anti-CD4 MAbs Q4120 and OKT4a (each 100 ng $well^{-1}$), respectively, in Tris-buffered saline, pH 7.2 containing 1% BSA and 0.25% gelatin were added to the wells. After overnight incubation at 25°C, bound HRP was quantified and bound mouse MAbs Q4120 and OKT4a were determined from subsequent binding of HRP-labelled anti-mouse IgG. The absorbance readings corresponding to bound HRP–gp120 and MAbs Q4120 and OKT4a in the absence of 3HP-β-LG were 0.652, 1.500 and 1.646, respectively.

Since the binding of 3HP-β-LG to CD4 was diminished at acidic pH, it was of interest to determine whether in such an environment 3HP-β-LG could still block the association between CD4 and the HIV-1 envelope glycoprotein gp120, such inhibition contributing to the anti-HIV-1 activity of 3HP-β-LG. Results shown in Fig. 2 indicate that 3HP-β-LG inhibited at pH 4 the binding to CD4 of not only gp120 but also of MAbs Q4120 and OKT4a specific for the gp120 binding site on CD4 (Sattentau *et al.*, 1989; Healey *et al.*, 1990), albeit at concentrations higher than those reported for pH 7 (Neurath *et al.*, 1996a).

3HP-β-LG is a ligand for soluble CD4 and for cellular CD4. This was shown by the binding of HeLa $CD4^+$ cells to immobilized 3HP-β-LG (Neurath *et al.*, 1996a). A decrease in pH from neutrality resulted in diminished binding of HeLa $CD4^+$ cells to magnetic beads coated with 3HP-β-LG. At pH 7, 5.5 and 5.0, 96, 90.2 and 48.3% of cells added to the beads became bound, respectively. Binding studies at pH <5 could not be carried out because the cells underwent lysis. 3HP-β-LG interfered with the binding of HeLa $CD4^+$ cells to anti-CD4 MAbs

Figure 3. Inhibitory effect of 3HP-β-LG on binding of HeLa CD4+ cells to magnetic beads coated with anti-CD4 antibodies.



HeLa CD4+ cells (10^6) suspended in Tris-buffered saline pH 7.2 or acetic acid/sodium acetate-buffered saline (pH 5 and 5.5), containing 10% normal chicken serum, were incubated with graded quantities of 3HP-β-LG for 30 min at 25°C and subsequently added to anti-CD4 magnetic beads. Bound and unbound cells were separated and quantified as described (Neurath *et al.*, 1996a).

linked to magnetic beads. As expected, this effect was also pH-dependent and was diminished at acidic pH (Fig. 3).

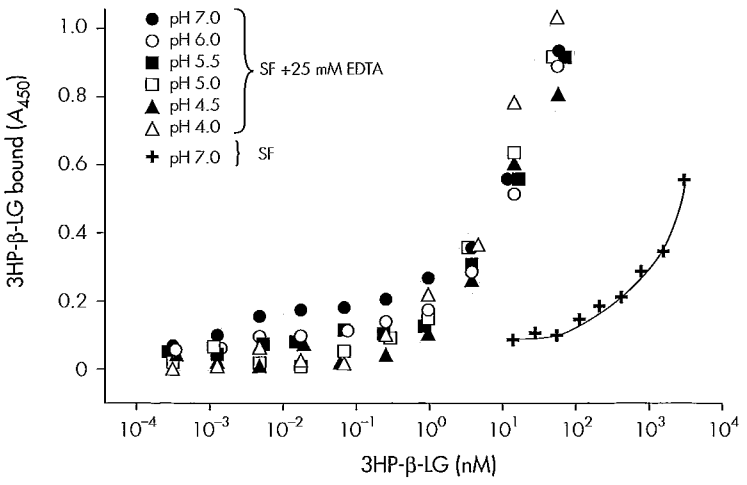
The results shown above indicate that 3HP-β-LG at nM concentrations, shown before to be sufficient to inhibit HIV-1 infection *in vitro* at neutral pH (Neurath *et al.*, 1996a, 1997), does not completely block HIV-1 binding sites on either soluble or cellular CD4 at acidic pH. This diminished activity is not due to inactivation of 3HP-β-LG at low pH since readjustment of pH after exposure of 3HP-β-LG to pH 4.0 for 30 min at 37°C did not diminish its inhibitory effect on gp120–CD4 association or its antiviral activity (data not shown). Further experiments revealed that 3HP-β-LG at concentrations ≥14 μM completely inhibited the interaction between gp120 with CD4 at pH 4.5. Direct measurements of anti-HIV-1 activity revealed that 3HP-β-LG added at pH 4 to 7.0 to cells 20 min before adding virus inhibited infection (EC_{50} 8.1±1.1 nM) independently of pH.

Effect of SF on 3HP-β-LG binding to CD4 and on its inhibitory effect on gp120–CD4 association

In accordance with the considerations proposed by the National Institute of Allergy and Infectious Diseases for the preclinical development of topical 'microbicides', we attempted to test the anti-HIV-1 activity of 3HP-β-LG in the presence of SF. SF proved to be toxic for tissue culture cell lines at dilutions 1:≤20. At dilutions of 1:≤40, SF had no effect on the anti-HIV-1 activity of 3HP-β-LG (data not shown). To assess the effect of SF at lower dilutions on 3HP-β-LG binding to CD4 and on its inhibitory effect on gp120–CD4 association, appropriate immunoassays were carried out. Initial experiments revealed that SF: (i) had a strong inhibitory effect on 3HP-β-LG binding to CD4 (97 and 50% inhibition of binding was observed at final SF dilutions of 1:1 and 1:100, respectively); (ii) inhibited at a 1:1 dilution binding of CD4+ HeLa cells to magnetic beads coated with 3HP-β-LG by 93.3%; and (iii) inhibited gp120–CD4 association (50% inhibition at an SF dilution of 1:25). These results suggested that the presence of SF has the potential to inhibit virus binding to CD4+ cells, and to interfere with the anti-HIV-1 activity of 3HP-β-LG. SF, owing to its buffering capacity, also increases the pH at the site for intended topical application of 3HP-β-LG (Voeller & Anderson, 1992). To explain the complex consequences of the presence of SF on HIV-1 infection of CD4+ cells, further studies were carried out.

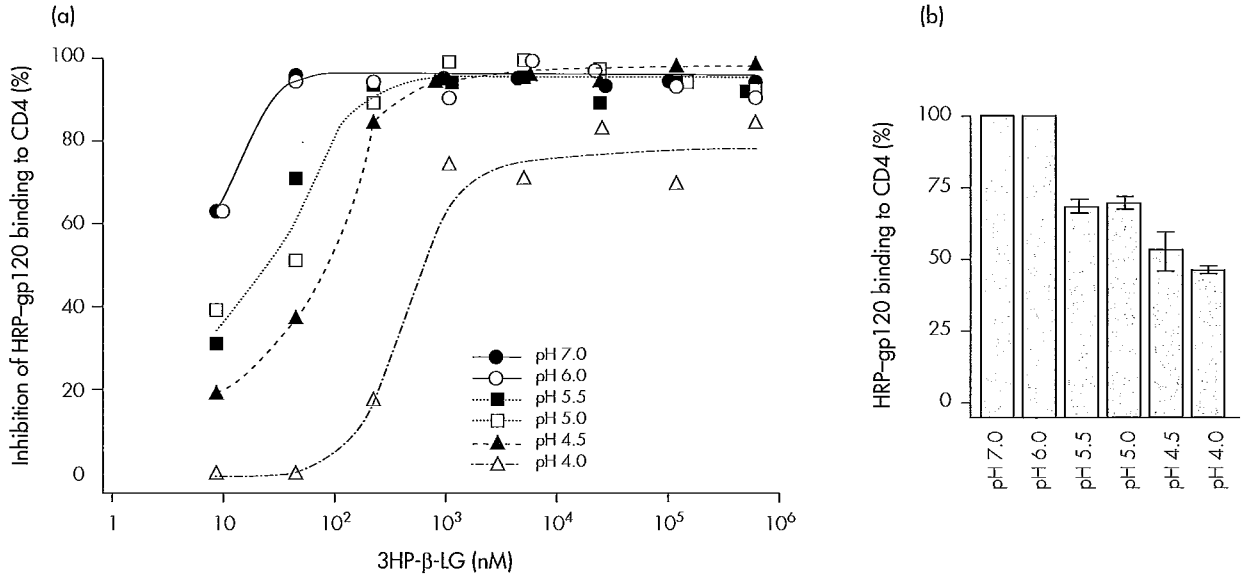
An ELISA test for CD4 was developed and applied to its determination in SF. CD4 (600 ng mL^{-1}) or an antigenically related molecule was detected in SF, in agreement with observations of Bagasra *et al.* (1988) and Scofield *et al.* (1992). When SF was submitted to affinity chromatography on immobilized 3HP-β-LG, CD4 (or the CD4-like protein detectable by ELISA) was quantitatively adsorbed to the column and subsequently eluted at pH 11.5 (data not shown). However, the decreased binding of 3HP-β-LG to CD4 could not be explained by the presence of low concentrations in SF of CD4 or a CD4-like molecule.

Figure 4. Binding of 3HP-β-LG to CD4 at different pH in the presence of SF.



Serial twofold dilutions of 3HP-β-LG in 0.1 M acetate buffers with different pH were incubated with SF (final dilution 1:4) containing 100 mM EDTA (final concentration 25 mM), adjusted by addition of acetic acid to the appropriate pH, for 30 min at 25°C. The mixtures were added to CD4-coated wells for 4 h at 25°C. The wells were washed and bound 3HP-β-LG was determined by ELISA using 1000-fold diluted rabbit antiserum against 3HP-β-LG, followed by HRP-labelled anti-rabbit IgG. EDTA was omitted in a control experiment at pH 7. Absorbance corresponding to wells to which 3HP-β-LG had not been added was 0.

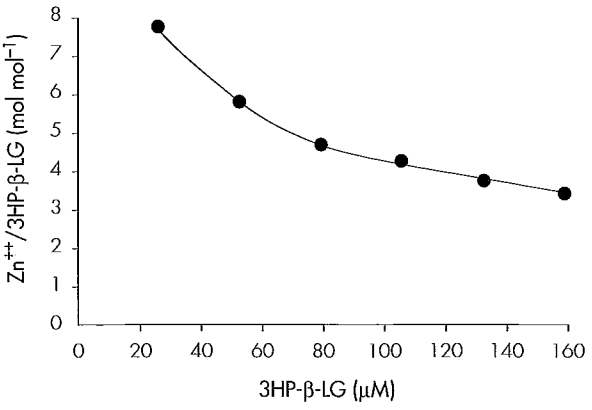
Figure 5. Effect of pH on gp120 binding to CD4-coated wells.



(a) Inhibitory effect of 3HP-β-LG on gp120 binding to CD4 at different pH in the presence of SF and EDTA. 75 µL of 0.1 M acetate buffers containing 1% BSA, 0.25% gelatin and graded quantities of 3HP-β-LG (to achieve final concentrations shown on the abscissa) were added to CD4-coated wells. After 30 min at 25°C, 25 µL of SF containing 0.1 M EDTA, adjusted to the appropriate pH, were added to the wells. After 4 h at 25°C, 10 µL of HRP–gp120 (10 µg mL^{-1}) were added to the wells. After overnight incubation, the wells were washed and bound HRP was quantified. The absorbance at 450 nm, corresponding to bound HRP–gp120 at pH 7 in the absence of 3HP-β-LG, was 0.695±0.023, as determined from duplicate experiments. The inhibitory effect of 3HP-β-LG was calculated considering the binding of HRP–gp120 to CD4 at each pH value in the absence of 3HP-β-LG as 100%. (b) The percentage of bound HRP–gp120 at different pH values considering the binding at pH 7 as 100%.

SF contains high levels of Zn^{++} (range 0.95–6.6 mM; Noack-Füller *et al.*, 1993). We considered the possibility that Zn^{++} might interfere with the binding of 3HP-β-LG to CD4. Results shown in Fig. 4 indicate that 3HP-β-LG at different pH binds to CD4 in the presence of EDTA much more effectively than in its absence, suggesting that

Figure 6. Binding of Zn^{++} to 3HP-β-LG.



Three mL of solutions containing 3HP-β-LG (0.5–3 mg mL^{-1} in 0.14 M NaCl, 10 mM HEPES pH 7.2) were mixed with 165 μL of 15.29 mM $ZnSO_4$. After 30 min at 25°C, the mixtures were centrifuged through a 10 kDa cut-off spin filter (UFV 2BGC10 Ultra-free-15 Centrifugal filter; Millipore). Zn^{++} in the filtrates was determined spectrophotometrically using the Zincon reagent, as described previously (Snell *et al.*, 1959). The amount of Zn^{++} recovered in the filtrates were subtracted from the amount of Zn^{++} originally added to 3HP-β-LG to calculate the amount of Zn^{++} in the retentates corresponding to Zn^{++} bound to 3HP-β-LG. In the absence of 3HP-β-LG, Zn^{++} was quantitatively recovered in the filtrates.

the complexing of Zn^{++} in SF by EDTA is essential for binding. In the absence of SF, EDTA did not affect 3HP-β-LG binding to CD4 (data not shown). Chelating of Zn^{++} in SF by EDTA also restored the inhibitory effect of 3HP-β-LG on gp120–CD4 association at different pH (Fig. 5) albeit at concentrations higher than those needed in the absence of SF. Thus, essentially complete inhibition of gp120–CD4 binding was accomplished at pH 4.5 to 7.0 at ≥1 μM concentrations of 3HP-β-LG. Less complete inhibition was observed at pH 4.0. These results suggest that as yet unidentified components of SF, in the absence of Zn^{++}, may partly interfere with CD4 blocking by 3HP-β-LG and that this problem can be overcome by simply increasing its concentration. The inhibitory effect of Zn^{++} can be ascribed to its binding to 3HP-β-LG (Fig. 6). The results of binding studies suggest the occurrence of several binding sites for Zn^{++} on 3HP-β-LG with different association constants since non-linear Scatchard plots were obtained by the analysis of experimental data.

Discussion

Sexual transmission is the most common route of infection by HIV-1 despite the fact that the virus is infrequently transmitted by sexual contact (Stratton & Alexander, 1994; Mayer & Anderson, 1995). It is anticipated that the rate of sexual HIV transmission could be substantially decreased by the use of topical chemical barrier methods (Alexander, 1996). Several compounds are being considered as constituents for such chemical barriers. They include spermicidal and microbicidal detergents, sulphated polysaccharides, compounds inhibiting cell growth and thereby replication of some viruses and compounds with an unknown mechanism of action (Alexander, 1996). As shown recently, chemical modification by 3HP of bovine β-LG, the major protein of whey, leads to the generation of a potent inhibitor of infection by HIV-1, including primary isolates belonging to distinct clades, macrophage-tropic viruses and distinct virus phenotypes (Neurath *et al.*, 1996a; AR Neurath, AK Debnath, N Strick, Y-Y Li, K Lin and S Jiang, unpublished results). 3HP-β-LG is also active against herpes simplex viruses type 1 and 2 (Neurath *et al.*, 1996b). Since β-LG can be produced inexpensively in large quantities in purified form and can be chemically modified on a large scale by a simple inexpensive procedure, 3HP-β-LG represents a favourable candidate to be considered for chemical barrier methods to prevent HIV-1 transmission.

The activity of antiviral compounds may be influenced by the environment in which they are expected to function (Kremer *et al.*, 1988; Dudley *et al.*, 1990; Bilello *et al.*, 1996). Therefore, it is advisable to carry out tests of antiviral compounds under conditions which would mimic the *in vivo* environment in which the antiviral activity is expected to be expressed. To the best of our knowledge, such tests have not been reported for the candidate compounds considered for prevention of sexual HIV-1 transmission. To prepare the background for future clinical evaluation of 3HP-β-LG, it was important to carry out such tests. It was possible to evaluate the effect of some anticipated environmental factors by measuring their effect on the protein–protein interactions contributing to the antiviral activity of 3HP-β-LG. The results of several ELISA assays revealed that at pH <7.0, corresponding to an acidic vaginal environment (Voeller & Anderson, 1992; Alexander, 1996), the binding of 3HP-β-LG to both soluble and cellular CD4 was decreased compared with binding observed at neutral pH. The presence of SF at different pH also contributed to decreased binding of 3HP-β-LG to CD4 and thus also to a decreased

inhibitory effect of 3HP-β-LG on gp120 binding to CD4. This effect could be partially attributed to Zn^{++} present in SF and to protein constituents binding to 3HP-β-LG. Neither the presence of SF nor of Zn^{++} (≥10 mM) resulted in elution of 3HP-β-LG preadsorbed to CD4, conditions which would mimic the *in vivo* application of 3HP-β-LG. Similarly, 3HP-β-LG once adsorbed to CD4-coated wells, was not eluted when exposed to pH 4.0 for 1 h at 37°C (data not shown). The decreased 3HP-β-LG–CD4 binding and the concomitant reduction of gp120–CD4 binding inhibition at low pH and/or in the presence of SF could be compensated for by an increase of the 3HP-β-LG concentration and by addition of EDTA to chelate Zn^{++} present in SF (Noack-Füller *et al.*, 1993). These two adjustments are economically and biologically feasible since 3HP-β-LG is inexpensive and when applied topically is non-toxic and non-irritating at concentrations ≤16 mg mL^{-1} (AR Neurath, AK Debnath, N Strick, Y-Y Li, K Lin and S Jiang, unpublished results). The decrease at acidic pH of the inhibition of gp120–CD4 association by 3HP-β-LG is also counterbalanced by inactivation of HIV-1 at pH <7 (Voeller & Anderson, 1992). Thus, the overall anti-HIV-1 activity of 3HP-β-LG appeared to be pH-independent. Nevertheless, results presented here suggest that future formulations of 3HP-β-LG should contain agents effectively chelating Zn^{++}, for example CaEDTA, which is expected to have fewer side effects than EDTA (disodium salt). CaEDTA was found to be equally effective as EDTA in chelating Zn^{++} and eliminating its interference with the inhibitory effect of 3HP-β-LG on gp120–CD4 association (data not shown).

Unlike SF, saliva had no effect on 3HP-β-LG binding to CD4 and on its interference with gp120–CD4 binding (data not shown). This observation is relevant to the intended application of 3HP-β-LG to prevent oral infection of new-borns by HIV-1, possibly contributing to mother-to-child virus transmission during birth.

In conclusion, results presented here provide background information necessary for the design of appropriate 3HP-β-LG formulations for *in vivo* applications.

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