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Research article

Anti-HIV-1 activity of anionic polymers: a comparative study of candidate microbicides

A Robert Neurath*, Nathan Strick and Yun-Yao Li

Address: Biochemical Virology Laboratory, The Lindsley F. Kimball Research Institute of the New York Blood Center, New York, NY 10021, USA

E-mail: A Neurath* - arneurath@worldnet.att.net; Nathan Strick - nathan_strick@nybc.org; Yun-Yao Li - yun-yao_li@nybc.org

*Corresponding author

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Abstract

Background: Cellulose acetate phthalate (CAP) in soluble form blocks coreceptor binding sites on the virus envelope glycoprotein gp120 and elicits gp41 six-helix bundle formation, processes involved in virus inactivation. CAP is not soluble at pH < 5.5, normal for microbicide target sites. Therefore, the interaction between insoluble micronized CAP and HIV-1 was studied. Carbomer 974P/BufferGel; carrageenan; cellulose sulfate; dextran/dextrin sulfate, poly(napthalene sulfonate) and poly(styrene-4-sulfonate) are also being considered as anti-HIV-1 microbicides, and their antiviral properties were compared with those of CAP.

Methods: Enzyme linked immunosorbent assays (ELISA) were used to (1) study HIV-1 IIIB and BaL binding to micronized CAP; (2) detect virus disintegration; and (3) measure gp41 six-helix bundle formation. Cells containing integrated HIV-1 LTR linked to the β-gal gene and expressing CD4 and coreceptors CXCR4 or CCR5 were used to measure virus infectivity.

Results: 1) HIV-1 IIIB and BaL, respectively, effectively bound to micronized CAP. 2) The interaction between HIV-1 and micronized CAP led to: (a) gp41 six-helix bundle formation; (b) virus disintegration and shedding of envelope glycoproteins; and (c) rapid loss of infectivity. Polymers other than CAP, except Carbomer 974P, elicited gp41 six-helix bundle formation in HIV-1 IIIB but only poly(napthalene sulfonate), in addition to CAP, had this effect on HIV-1 BaL. These polymers differed with respect to their virucidal activities, the differences being more pronounced for HIV-1 BaL.

Conclusions: Micronized CAP is the only candidate topical microbicide with the capacity to remove rapidly by adsorption from physiological fluids HIV-1 of both the X4 and R5 biotypes and is likely to prevent virus contact with target cells. The interaction between micronized CAP and HIV-1 leads to rapid virus inactivation. Among other anionic polymers, cellulose sulfate, BufferGel and aryl sulfonates appear most effective in this respect.

Background

Several anionic polymers were shown to have HIV-1 inhibitory activity and are being considered and evaluated as vaginal topical microbicides for prevention of transmission of sexually transmitted disease (STD) pathogens, including HIV-1. These polymers belong to several classes: (1) sulfate esters of polysaccharides (dextrin and dextran sulfates [1–11]; cellulose sulfate [12–15]; sulfate esters of

galactose + 3,6-anhydrogalactose copolymers {carrageenan; [1,16–19]}); (2) polymers containing sulfonated benzene or naphthalene rings {poly(styrene-4-sulfonate) [20,21]} and naphthalene sulfonate polymers {PRO2000 [22–24]}; and (3) polycarboxylates (acrylic acid polymers {Carbomer 974P; BufferGel [25–29]}); and acetyl phthaloyl cellulose {cellulose acetate phthalate (CAP) [30–37]}.

The mechanisms involved in sexual transmission of HIV-1 appear to be complex and are not yet fully defined [38,39]. Thus, a microbicide with HIV-1 inhibitory activity could provide protection *in vivo* only if both the compound and the virus reach susceptible cells before productive infection could occur. This might be difficult to accomplish. For this reason, it is preferable that microbicides irreversibly inactivate the virus upon the first compound/HIV-1 encounter in the vaginal environment, i.e. microbicides should have direct virucidal activity. Such activity was reported for dextran sulfate after 2 h incubation with HIV-1 [2] but results of other studies indicated that dextran sulfate failed to neutralize virus infectivity [4,10]. Inactivation of HIV-1 by carrageenan was reported in a review article [18]. BufferGel is virucidal against HIV-1 by providing a buffered low pH environment (pH 3.9 [25]), but virus inactivation might be incomplete and possibly compromised by minor pH increases due to the presence of seminal fluid [40]. To be effective in preventing infection by direct virus inactivation, anti-HIV-1 microbicides must be virucidal against both X4 and R5 viruses, utilizing CXCR4 and CCR5 as coreceptors [39,41], respectively, R5 HIV-1 being most frequently sexually transmitted. Recent results [36,37], (R. Shattock, personal communication) indicate that CAP in soluble form meets these requirements. Since CAP, unlike the other polymers listed above, is not soluble at low pH, characteristic for a normal vaginal environment [42], micronized forms of CAP had to be used in microbicidal formulations of this compound [30–34]. Therefore, it was of interest to determine how CAP in micronized form and other candidate polymeric microbicides affect X4 and R5 viruses represented by HIV-1 IIIB and BaL, respectively.

Methods

Reagents

Aquateric (the micronized form of CAP containing ≈ 66% CAP and ≈ 34% of Poloxamer and distilled acetylated monoglycerides) was obtained from the FMC Corporation, Philadelphia, PA. Cellulose acetate phthalate (CAP) was a gift from Eastman Chemical Company, Kingsport, TN, and micronized using a SPEX 6800 freezer mill (SPEX CertiPrep, Metuchen, NJ) with the following settings: precooling time 10 min, cooling time 1 min, input frequency 15; 3 cycles of 3 min each. The following additional polymers were obtained from commercial sources likely to be different from those for proprietary products being developed as microbicides: carrageenans κ and λ (Sigma, St. Louis, MO; mixed at a 1:1 (w/w) ratio in all experiments); cellulose sulfate (Across Organics, Piscataway, NJ); dextran sulfate (M.W. 500,000; Pharmacia, Kalamazoo, MI); poly(naphthalene sulfonate) (BASF, Parsippany, NJ); poly(styrene-4-sulfonate) (Polysciences, Inc., Warrington, PA); and Carbomer 974P (B. F. Goodrich, Cleveland, OH). Poloxamer {an α-Hydro-γ-hydroxypoly(oxyethylene)poly(oxypropylene)poly(oxyethylene) block copolymer} was from Spectrum, New Brunswick, NJ. Goat serum was from Atlanta Biologicals, Norcross, CA. BufferGel was a gift from Dr. Kevin J. Whaley, John Hopkins University. The mouse monoclonal antibody (mAb), NC-1, raised against the gp41 six/helix bundle from HIV-1 IIIB was prepared as described [43] and shown to react also with six-helix bundles from HIV-1 BaL [37]. Rabbit antibodies against the gp41 six-helix bundle and against HIV-1 IIIB gp120 were prepared as described [44,45]. The latter antibodies were shown to crossreact with HIV-1 BaL gp120 [37]. Rabbit antibodies against HIV-1 tat as well as recombinant HIV-1 IIIB gp120 and biotinylated soluble CD4 (sCD4) were from ImmunoDiagnostics, Inc., Woburn, MA. Horseradish peroxidase (HRP) labeled streptavidin was from Zymed, South San Francisco, CA. HRP labeled goat anti-rabbit IgG was from Southern Biotechnology Associates, Inc., Birmingham, AL. HRP was quantitated using a kit from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD. Biotin labeled mouse IgG was obtained from the Roche Diagnostics Corporation, Indianapolis, IN. Recombinant protein A/G was from Pierce, Rockford, IL. Enzyme linked immunosorbent assay (ELISA) kits for the HIV-1 p24 antigen were from Beckman Coulter, Inc., Miami, FL. Pelletted, 1000-fold concentrates of HIV-1 IIIB (6.8×10^{10} virus particles/ml) and BaL (2.47×10^{10} virus particles/ml) [46] were from Advanced Biotechnologies, Inc., Colombia, MD. Human seminal fluid was purchased from New England Immunology Associates, Cambridge, MA. HeLa-CD4-LTR-β-gal and MAGI-CCR5 cells were obtained from the AIDS Research and Reference Reagent Program contributed by Drs. M. Emerman and J. Overbaugh. Dulbecco's modified Eagle medium (DMEM) was from GIBCO™ Invitrogen Corporation, Carlsbad, CA. The Galacto-Light Plus chemiluminescence reporter assay for β-galactosidase was from Applied Biosystems, Foster City, CA.

Citrate/phosphate buffers in the pH range of 2.9 – 5.0 were prepared as described [47].

Enzyme-linked immunosorbent assays (ELISA)

The sandwich ELISA for gp41 six-helix bundles was performed as described [37,45]. HIV-1 IIIB and BaL virus preparations, respectively, treated with distinct anionic polymers, were precipitated with 3% polyethylene glycol

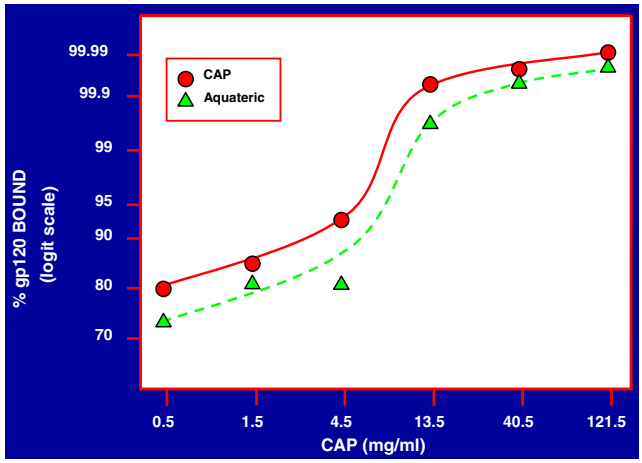

Figure 1
Binding of HIV-1 IIIB gp120 to micronized CAP and Aquateric, respectively. Graded quantities of micronized CAP and Aquateric, respectively, in 0.5 ml of 0.14 M NaCl were mixed with 100 μl of a solution containing 1 μg of HIV-1 IIIB gp120, and 10 μg each of bovine serum albumin (BSA) and gelatin. After incubation for 5 min at 37°C the mixtures were cooled on ice, centrifuged at 10,000 rpm and the supernatant fluids were filtered through 0.45 μ filters which had been pre-washed with a solution containing 100 μg/ml of each BSA and gelatin in 0.14 M NaCl. gp120 in the filtrates was determined as described in the Methods section. The percentage of gp120 recovered in the filtrates was determined from calibration curves relating absorbance readings to the concentration of gp120.

6000 (PEG) in order to separate virus particles from excess compounds. The pellets were dissolved in lysis buffer [1% Nonidet P40 {NP40}, 100 μg/ml bovine serum albumin (BSA) in phosphate buffered saline (PBS)], incubated for 30 min at 20°C and then added to wells coated with rabbit polyclonal antibodies to the gp41 core. Control virus was treated similarly. The lysis buffer was used as a safety precaution to minimize work with potentially infectious virus. Similar results were obtained when PBS was used for resuspension of virus particles instead of lysis buffer. After incubation at 4°C overnight, the bound six-helix bundles were detected by adding mAb NC-1 (1 μg/ml in PBS/1% BSA/1% gelatin {100 μl/well} for 1 h at 37°C. Subsequently the wells were washed three times with PBS/0.05% Tween 20, and biotin labeled anti-mouse IgG (100 μl/well; 125 ng/ml diluted in PBS containing 1% dry fat-free milk) was added. After incubation for 1 h at 37°C the wells were washed and HRP-streptavidin (125 ng/ml in PBS containing 10% goat serum; 100 μl/well) was added. After incubation for 1 h at 37°C, the wells were washed six times with PBS/0.05% Tween 20. Bound HRP was detected using the test kit from Kirkegaard & Perry Laboratories following the manufacturer's protocol, and the absorbance was read at 450 nm.

Recombinant gp120 HIV-1 IIIB in the presence of a 10-fold excess of BSA and gelatin, respectively, was quantitated by adding the gp120/BSA/gelatin mixtures (before and after binding to micronized CAP or Aquateric; Fig. 1) at serial 5-fold dilutions (2-fold to 3.9 × 10^6-fold in 0.14 M NaCl-0.01 M Tris {TS}) to 96-well polystyrene plates (Immulon II, Dynatech Laboratories, Inc. Chantilly, VA). After overnight incubation at 4°C, the wells were washed and rabbit anti-gp120 (diluted 1:1,000 in 20% goat serum – 0.05% Tween 20 in PBS pH 7.4) was added. After 2 h at 37°C the wells were washed and HRP labeled goat anti-rabbit IgG (diluted in 1% BSA – 0.25% gelatin – 0.05% Tween 20 in PBS pH 7.4; {250 ng/well}) was added. After 2 h at 37°C bound HRP was detected using the kit from Kirkegaard & Perry Laboratories. The quantity of gp120 not adsorbed to CAP/Aquateric was determined from calibration curves relating absorbance readings to the concentration of gp120 not exposed to CAP/Aquateric.

The envelope glycoprotein gp120 released from HIV-1 BaL was quantitated as follows: Wells of polystyrene plates were first coated with protein A/G (1 μg/well) in 0.1 M Tris buffer, pH 8.8 for 2 h at 20°C followed by polyclonal rabbit anti-gp120 (diluted 500-fold) for 1 h at 20°C. Serial 2-fold dilutions in PBS containing 1% BSA and 1% gelatin (PBS-BG) of preparations containing gp120 were added to the wells. After 4 h at 20°C the wells were washed and bound gp120 was detected by addition of biotinyl sCD4 (1 μg) followed by HRP streptavidin. Bound HRP was quantitated as described above. The experiments were done in triplicate.

Measurements of HIV-1 infectivity

Two-fold serial dilutions of HIV-1 IIIB treated with distinct anionic polymers, and separated from these polymers by precipitation with 3% PEG or by centrifugation at 14,000 rpm for 1 h, and control virus (100 μl), respectively, were added to HeLa-CD4-LTR-β-gal cells which had been plated a day before infection in 96-well plates at 1 × 10^4 cells/well in 100 μl of DMEM medium containing 10% fetal bovine serum (FBS). In some experiments, anti-HIV-1 tat IgG (25 μg) was added to treated and control virus to suppress the effect of extracellular tat, which may have been present in the virus preparations, on virus infection [48,49]. After incubation at 37°C for 48 h, the culture supernatant fluids were removed and the cells washed once with PBS. Subsequently, 50 μl of lysis buffer from the Galacto-Light Plus kit were added to the wells for 1 h at 20°C. Aliquots (20 μl) of the cell lysates were transferred into wells of 96-well microplates and β-galactosidase was quantitated using the Galacto-Light Plus System chemiluminescence reporter assay in a Microlight ML 2250 luminometer (Dynatech Laboratories, Inc. Chantilly, VA). The infectivity of treated and control HIV-1 BaL was measured

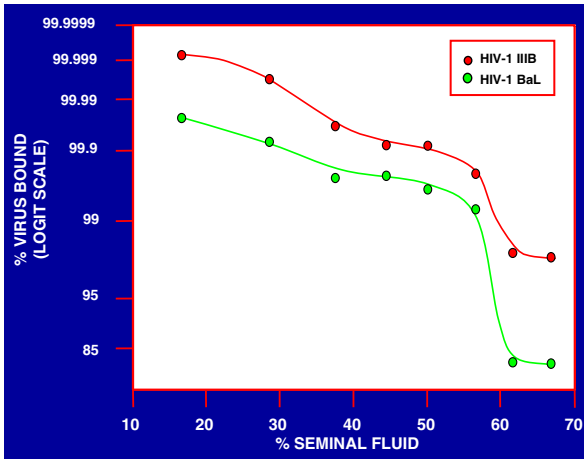

Figure 2
Binding of HIV-1 IIIB and BaL, respectively, to Aquateric in the presence of human seminal fluid. Five hundred μl aliquots of a formulation containing Aquateric (18% Aquateric, 6% hydroxypropyl methylcellulose, 76% glycerol) were mixed with graded volumes of seminal fluid (range: 100 μl – 1,000 μl) each containing equal amounts of purified HIV-1 IIIB (6.8×10^9 particles) and BaL (2.5×10^9 particles), respectively. After incubation for 5 min at 37°C the mixtures were cooled in ice, centrifuged at 10,000 rpm for 10 min in order to pellet Aquateric. The supernatant fluids were all adjusted to identical volumes (1.5 ml) and NP40 was added to a final concentration of 1%. After incubation for 30 min at 37°C, serial 5-fold dilutions (1/1,000 to $1/3.9 \times 10^8$) were prepared and p24 antigen in each dilution was measured by ELISA. The percentage of p24 antigen (representing a relative measure of the number of HIV-1 virus particles) was determined from calibration curves relating absorbance readings to the concentration of p24 antigen.

by the same method except that MAGI-CCR5 cells were used.

Measurement of HIV-1 infectivity in seminal fluid is impeded by the cytotoxicity of seminal plasma [50,51]. contributed to by spermine [52]. Such effects were minimized by using for titrations of virus infectivity pellets after precipitation with 3% PEG or after centrifugation (14,000 rpm for 1 h).

Results

Micronized CAP induces gp41 six-helix bundle formation and is virucidal against HIV-1

Earlier studies [36,37]. indicated that soluble CAP at neutral pH binds to the CXCR4 and CCR5 coreceptor binding sites on HIV-1 IIIB and BaL, respectively, and induced the formation of "dead-end" gp41 six-helix bundles. These two properties of soluble CAP appear to be involved in the virus inhibitory and virucidal activities of this compound. However, CAP is not soluble at < pH 5.5 and would precipitate in a healthy human vaginal environment which is mildly acidic with a pH range of 3.5 – 4.5 [53]. The precipitate would have drastically reduced antiviral activity. To overcome this problem, CAP in a micronized form was used and proven to have virucidal activity against HIV-1 IIIB [31] and shown to be effective in reducing vaginal infection by simian immunodeficiency virus (SIV) in a macaque model [34]. Micronized CAP provides a low pH buffering system and this may have contributed to its virucidal properties. In order to correlate the properties of CAP in soluble and micronized forms, it was necessary to establish whether or not micronized CAP would bind HIV-1 virus particles and HIV-1 envelope glycoproteins, and induce the formation of gp41 six-helix bundles, a marker of irreversible virus inactivation, as did soluble CAP [37]. First it was established that HIV-1 gp120 bound to micronized CAP (provided in the form of micronized CAP particles or of a commercial product, "Aquateric", containing about 66% of CAP and used for enteric coating of tablets and capsules in the pharmaceutical industry [54]); (Fig. 1). Similar results were obtained when gp120 was diluted in seminal fluid (diluted 1:1 with 0.14 M NaCl). Under these conditions, ≥ 99.9997% of gp120 bound to micronized CAP and Aquateric (final concentration of CAP: 121.5 mg/ml), respectively. HIV-1 IIIB and BaL virus particles also bound effectively in the presence of seminal fluid to Aquateric formulated into a topical gel (Fig. 2).

As a result of interaction with micronized CAP (in the form of Aquateric), the integrity of both HIV-1 IIIB and HIV-1 BaL virus particles was strikingly affected as indicated by the observation that most of the p24 antigen after virus contact with micronized CAP was no longer precipitable by 3% polyethylene glycol 6000 (PEG) unlike p24 antigen within untreated virus particles which precipitates under these conditions (Fig. 3). Furthermore, interaction with micronized CAP elicited the formation of gp41 six-helix bundles which were undetectable or present at low levels in untreated virus particles [37]. The six-helix bundles detectable by ELISA were mostly not precipitable by 3% PEG indicating that they were no longer associated with virus particles (Fig. 3). Furthermore, they were detected partially (HIV-1 IIIB) or entirely (HIV-1 BaL) in a form not bound anymore to the micronized CAP particles, unlike most of the virus-derived p24 antigen. This suggests that interaction of virus particles with micronized CAP resulted in stripping off of the viral envelopes which was more noticeable with HIV-1 BaL than with HIV-1 IIIB.

To confirm that gp41 six helix bundle formation was caused by contact of virus particles with the micronized form of CAP (= Aquateric) and not by low pH contributed by CAP, purified HIV-1 III B and BaL, respectively, was exposed for 5 min at 37°C to acidic buffers (final pH 2.9, 3.5, 4.0, 4.5 and 5.0). The suspensions were neutralized

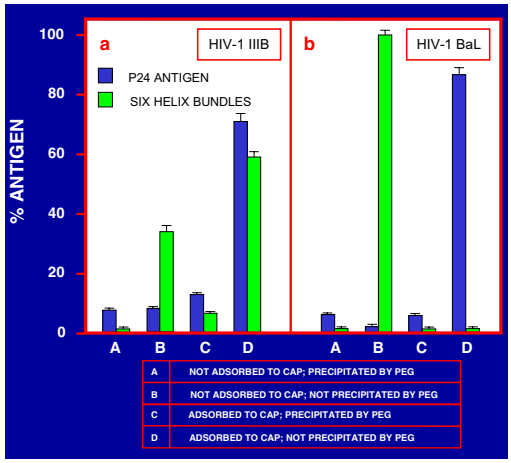

Figure 3
Aquateric causes the disintegration of HIV-1 IIIB and BaL virus particles and induces the formation of gp41 six-helix bundles. Equal volumes of HIV-1 IIIB and BaL virus preparations (6.8×10^{10} and 2.47×10^{10} virus particles, respectively, per ml) and the Aquateric formulation (see legend to Fig. 2) were mixed and incubated for 5 min at 37°C. Aquateric was pelletted by centrifugation at 10,000 rpm for 10 min and the supernatant fluids were neutralized by addition of 1 M $Na_2HPO_4$and filtered through 0.45 μ filters. The pellets containing Aquateric and adsorbed virus antigens were resuspended and brought to pH 7.0 by addition of 1 M Na_3HPO_4. The supernatant fluids and the resuspended pellets, respectively, were mixed with a solution of PEG 6000 (final concentration 3%). The supernatant fluids and the resuspended pellets after PEG precipitation were mixed with NP40 (final concentration 1%). After incubation for 30 min at 37°C and 20°C, respectively, p24 antigen and six-helix bundles in the supernatant fluids and resuspended pellets were measured by ELISA.

and assayed by ELISA for six-helix bundles. The results were negative (absorbance in the 0 to 0.068 range, the same as for viruses exposed to pH 7.0 in the absence of CAP). Poloxamer, a constituent of Aquateric (≤ 33%) at a final concentration of 30 mg/ml also did not induce the formation of six-helix bundles.

Results shown above indicated that HIV-1 adsorbed onto micronized CAP and that the CAP – virus interaction lead to the formation of gp41 six-helix bundles, a hallmark for HIV-1 inactivation. [37] To confirm that contact of virus with micronized CAP leads to loss of HIV-1 infectivity, HIV-1 IIIB and BaL, respectively, were diluted in seminal fluid and the diluted virus preparations were mixed with an Aquateric formulation (for composition, see legend to Fig. 2) at volume ratios of 3:1, 1:1 and 1:3 for 5 min at 37°C. Subsequently, the micronized Aquateric particles were sedimented by centrifugation. The supernatant fluids were brought to pH 7.0 and the pellets were dissolved by addition of Na_3PO_4 (final pH = 7.0). All fractions were then precipitated by 3% PEG and the redissolved pellets tested at 2- to 256-fold dilutions for infectivity (see Methods section). Infectious virus was undetectable (≥ 99.6% and ≥ 99.2% inactivation of HIV-1 IIIB and BaL, respectively). Similar results were obtained with other CAP formulations (data not shown).

Induction of six-helix bundle formation by polymeric anionic candidate microbicides

Since elicitation of gp41 six-helix bundles might be involved in, or contribute to the virucidal activity of candidate microbicides, it was of interest to determine whether anionic polymers other than CAP also induced six-helix bundle formation in HIV-1 IIIB and BaL virus particles.

Results in Fig. 4 show that carrageenan, cellulose sulfate, dextran sulfate, poly(napthalene sulfonate) and poly(styrene-4-sulfonate) all elicit gp41 six-helix bundle formation in HIV-1 IIIB but somewhat less effectively in comparison with CAP. On the other hand, Carbomer 974P, the active ingredient in BufferGel, was inactive in this respect. Strikingly different results were obtained for HIV-1 BaL (Fig. 5), and show that only CAP and to a lesser extent poly(napthalene sulfonate) elicited gp41 six-helix bundles detectable by ELISA.

Effect of anionic polymeric microbicide candidates on the integrity of HIV-1 particles

Since micronized CAP caused the dissociation of HIV-1 particles [31]; (Fig. 3), it was of interest to determine whether anionic polymeric candidate microbicides in soluble form at neutral pH would have similar effects. While no such effects were seen with HIV-1 IIIB, the integrity of HIV-1 BaL appeared to be partially diminished by treatment with CAP and drastically affected by cellulose sulfate (Fig. 6). The latter finding was confirmed by the observation that HIV-1 BaL gp120 was no longer precipitable by 3% PEG or pellettable by centrifugation after treatment of the virus with cellulose sulfate, unlike gp120 associated with untreated virus particles (Fig. 7). This was not seen with cellulose sulfate treated HIV-1 IIIB (data not shown).

Comparative virucidal activity of polymeric anionic microbicide candidates

HIV-1 IIIB and BaL, respectively, were treated with the distinct polymers (10 mg/ml) for 5 min at 37°C and the infectivity of the treated viruses was compared with that of control untreated HIV-1. In repeated experiments with HIV-1 IIIB, soluble and micronized CAP, cellulose sulfate, poly(naphthalene sulfonate), poly(styrene-4-sulfonate) and BufferGel, respectively, caused a ≥ 140-fold reduction in infectivity, while dextran sulfate, carrageenan and Carbomer 974P (pH 7), respectively, were less effective (4- to

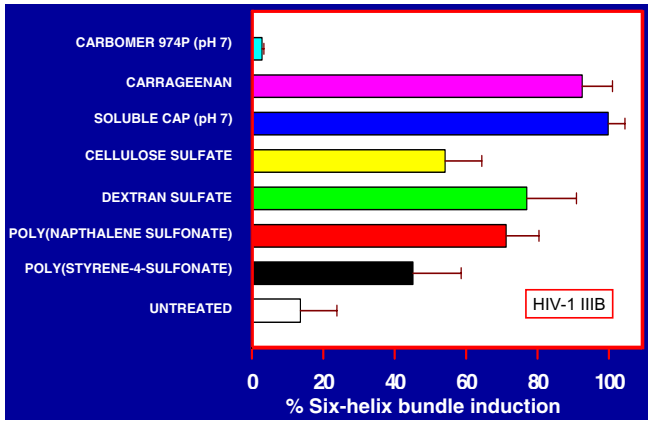

Figure 4
gp41 six-helix bundle induction in HIV-1 IIIB by distinct anionic polymers representing candidate microbicides. Purified HIV-1 IIIB (7×10^9 particles in TS) was precipitated with 3% PEG. The pellets were resuspended in 100 μl of phosphate buffer saline (PBS) to separate the virus from soluble HIV-1 antigens in the preparation. The different anionic polymers (listed on the ordinate) were added to a final concentration of 5 mg/ml except for Carbomer 974P (2.5 mg/ml). After incubation for 5 min at 37°C, the mixtures were cooled in ice and PEG was added (final concentration 3%). The pellets after centrifugation were washed once with 3% PEG in PBS, repelletted and finally resuspended in PBS containing 1% NP40 and 100 μg/ml BSA and incubated at 20°C for 30 min. The formation of six-helix bundles was determined by ELISA (see Methods section). All experiments were done in triplicate, the absorbance readings are shown as percentages of the absorbance corresponding to six-helix bundles elicited by CAP (= 100%).

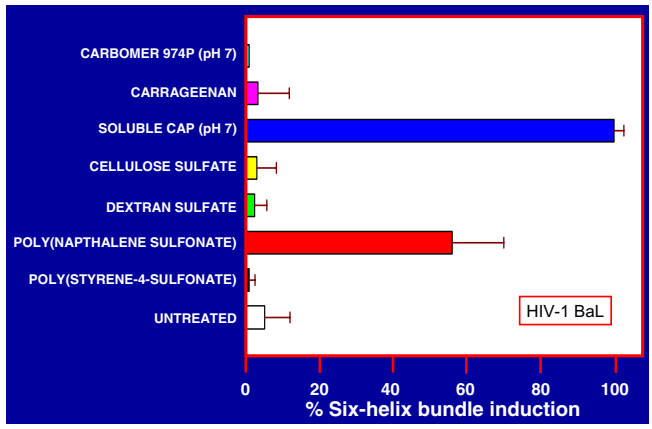

Figure 5
gp41 six-helix bundle induction in HIV-1 BaL by distinct anionic polymers respresenting candidate microbicides. Purified HIV-1 BaL (2.5×10^9 particles in Dulbecco's modified Eagle medium {DMEM} + 20% fetal bovine serum {FBS}) were precipitated with 3% PEG and subsequently treated as described in the legend for Fig. 4.

25-fold reduction in infectivity). A typical example for HIV-1 IIIB is shown in Fig. 8.

Representative results shown in Fig. 9 indicate that the polymers differed from each other more substantially with respect to their capacity to inactivate HIV-1 BaL, micronized CAP, cellulose sulfate, BufferGel and the aryl sulfonates being most effective.

Inhibitory effect of polymeric anionic microbicide candidates on blood clotting

For the selection of the most promising candidate microbicides, it is important to compare not only their virus inhibitory and virucidal activities, but to also study their potential untoward effects. Since heparin and other anionic polymers are known to inhibit blood clotting [55], the effect of anionic polymeric microbicide candidates on blood clotting was measured. While CAP, carrageenan and BufferGel (Carbomer 974P) had moderate or no effects, cellulose sulfate, dextran sulfate, poly(napthalene sulfonate) and poly(styrene-4-sulfonate) greatly inhibited clotting of ACD plasma (Fig. 10). The increase in clotting time might be potentially undesirable for topical applications under conditions of local preexisting injury or bleeding.

Discussion

Several anionic polymers are being considered as topical microbicides to prevent sexual transmission of HIV-1. They include: (1) sulfate esters of polysaccharides {carrageenan [1,16–19], cellulose sulfate [12–15], and dextran or dextrin sulfate [1–11]}; (2) aryl sulfonates {poly(naphthalene sulfonate) [22–24] and poly(styrene-4-sulfonate) [20,21]}; and (3) aliphatic {Carbomer 974P/BufferGel [25–29]} and aromatic carboxylates {CAP [30–37]}. The anti-HIV-1 properties of these polymers are expected to depend on their complexation with virus proteins which is likely to occur mainly through the formation of salt linkages or ion pairs between oppositely charged groups on the polymers and virus proteins, respectively [56]. However, the anti-HIV-1 properties of these polymers may depend not only on their charge density, but also on the characteristics of their uncharged portions, which may be involved in hydrophobic and hydrogen bonding interactions which can elicit profound conformational changes in hydrophobic regions of proteins and cause disassembly of complex proteins consisting of subunits [57]. For these reasons it is not be expected that all anionic

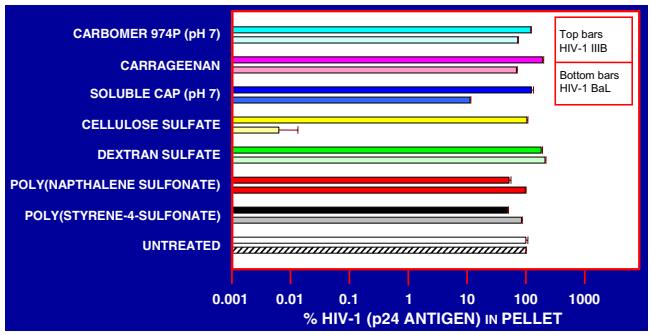

Figure 6
Effect of anionic polymeric microbicide candidates on the integrity of HIV-1 IIIB and BaL virus particles. Purified HIV-1 IIIB (7×10^9 particles/100 μl) and BaL (2.5×10^9 particles/100 μl) untreated or treated with the polymeric substances shown on the abscissa (final concentrations 5 mg/ml except for Carbomer 974P {2.5 mg/ml}) for 5 min at 37°C. The mixtures were cooled on ice and the virus particles were pelletted by centrifugation (1 h at 14,000 rpm in a Beckman 5415 C microfuge). The pellets were resuspended in lysis buffer (1% NP40, 100 μg/ml BSA in PBS) and incubated for 30 min at 37°C. p24 antigen was determined in serial dilutions of the lysates by ELISA. The levels of p24 antigen in the treated virus preparations were compared with those corresponding to lysed untreated virus (= 100%).

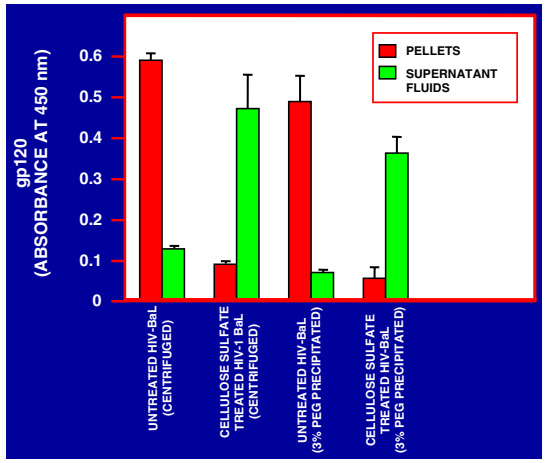

Figure 7
Release of gp120 from HIV-1 BaL by treatment with cellulose sulfate. Purified HIV-1 BaL (2.5×10^9 particles/100 μl) was treated with cellulose sulfate (final concentration 5 mg/ml) for 5 min at 37°C. The mixture was cooled in ice and divided into 2 aliquots. One aliquot was centrifuged for 1 h at 14,000 rpm and the 2nd aliquot was precipitated with PEG 6000 (final concentration 3%). gp120 in the respective supernatant fluids and pellets (resuspended in PBS) was determined by ELISA as described [37]; (see Methods section).

polymers, considered as anti-HIV-1 microbicides, would have similar biological activities.

In vivo protection by anti-HIV-1 compounds against sexual transmission of the virus could only be accomplished if the compounds at sufficient concentrations, and the virus both reach susceptible cells within genital mucosa before productive infection could occur [38,39]. Considering the complex events involved in sexual transmission of HIV-1, this might not be easily accomplished. Therefore, inactivation of HIV-1 infectivity preceding virus contact with susceptible target cells should be considered a preferred mechanism of protection against infection. For this reason it was of interest to study the virucidal activity of anionic polymeric microbicide candidates against both X4 (using CXCR4 as coreceptor) and R5 (using CCR5 as coreceptor) viruses, the latter being most frequently transmitted sexually [39].

Soluble CAP at neutral pH was shown to bind strongly to the coreceptor binding sites on gp120 of both X4 and R5 viruses [36] and to elicit the formation of gp41 six-helix bundles, the hallmark of irreversible virus inactivation [37]. Thus, HIV-1 exposed to CAP at sufficient concentrations is rendered noninfectious, and continued presence of CAP is not required to maintain lack of infectivity. CAP differs from all other anionic polymers listed above, in that it is not soluble at pH < 5.5. Therefore, CAP would be expected to precipitate in a normal acidic vaginal environment. To avoid this, CAP in micronized form (which does not aggregate at low pH), instead of CAP in soluble form is being considered as a topical microbicide [30–35]. Micronized CAP (Aquateric) was shown to be virucidal against HIV-1, herpesviruses and several nonviral sexually transmitted disease (STD) pathogens [30–34]. The virucidal activity of micronized CAP could at least partly be explained by its buffering capacity at low pH [40] since it is a free acid while other anionic polymeric microbicide candidates (except BufferGel, the active ingredient of which is Carbomer 974P [27]) are sodium salts. Results presented here explain how micronized CAP inactivates HIV-1. Both the envelope glycoprotein gp120 (Fig. 1) and HIV-1 IIIB and BaL virus particles (Fig. 2) in the presence of an excess of nonviral proteins, including seminal fluid, bind effectively to micronized CAP. Under *in vivo* conditions (when the virus concentrations are by one to four orders of magnitude lower than those used here under experimental conditions [58,59], (Fig. 2)), this would be expected to physically remove virus particles by adsorption onto micronized CAP (Aquateric) so that they would cease to be available to initiate the complex sequence of events leading to mucosal infection. Furthermore, interac-

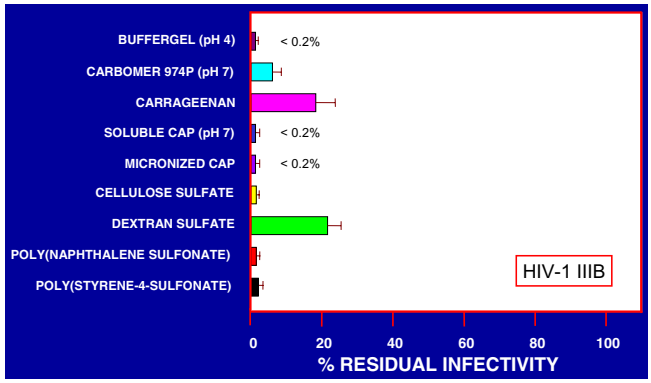

Figure 8
Inactivation of HIV-1 IIIB by polymeric anionic candidate microbicides. HIV-1 IIIB (7×10^9 virus particles/100 μl) was treated with the compounds indicated on the abscissa (final concentrations 10 mg/ml except for Carbomer 974P [2.5 mg/ml; neutralized to pH 7.0; at higher concentrations the solutions became too viscous to handle]) for 5 min at 37°C. The final dilution of BufferGel was 4-fold (final pH = 4.0). The preparations were cooled on ice and virus particles were separated from the polymers and their infectivity determined as described in the Methods section. For comparison, results obtained with a formulation of micronized CAP (= Aquateric; see legend for Fig. 2) are shown.

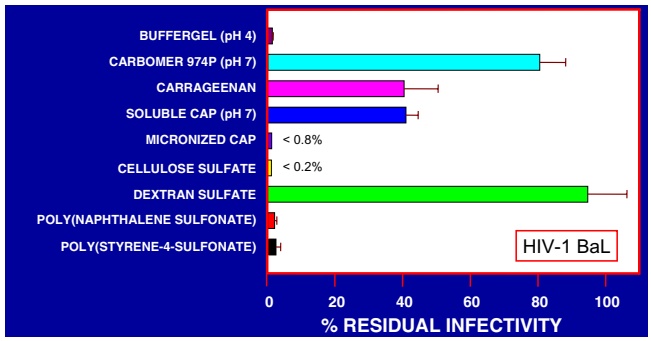

Figure 9
Inactivation of HIV-1 BaL by polymeric anionic candidate microbicides. HIV-1 BaL (2.5×10^9 virus particles/100 μl) was treated as described in the legend for Fig. 8.

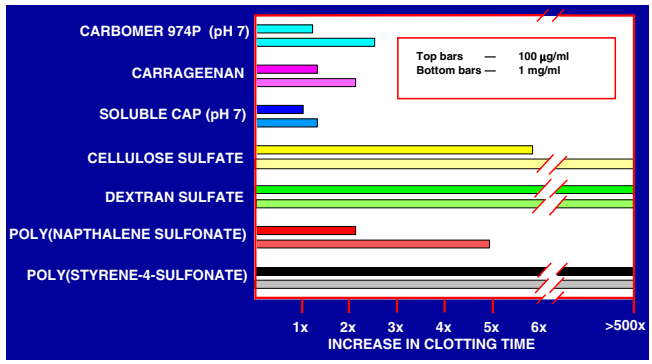

Figure 10
Effect of polymeric anionic candidate microbicides on clotting of ACD plasma. The compounds indicated on the abscissa (final concentrations of 100 and 1000 μg/ml, respectively) were added to fresh citrated plasma prewarmed to 37°C. $CaCl_2$ was added to a final concentration of 10 mM, and the time when clot formation had started was measured. The increase in clotting time as compared with the time required for clotting of plasma in the absence of any added compounds is shown.

tion with micronized CAP elicits the formation of gp41 six-helix bundles and causes the stripping off of HIV-1 envelope glycoproteins (Fig. 3). The formation of gp41 six-helix bundles was not due to exposure to a low pH environment provided by the micronized CAP, since exposure to low pH buffers did not elicit gp41 six-helix bundle formation. In summary, the contact of HIV-1 IIIB and HIV-1 BaL, representatives of X4 and R5 viruses respectively, with micronized CAP, resulted in a complex sequence of events involving virus adsorption, exposure to low pH, induction of gp41 six-helix bundles, and stripping off of viral envelopes and virus disintegration, ultimately resulting in loss of infectivity.

In order to contribute to the understanding of the potential virucidal activity of anionic polymers, their capacity to induce gp41 six-helix bundle formation was measured and compared with that of CAP in soluble form (pH 7.0). All polymers except Carbomer 974P elicited gp41 six-helix bundle formation in HIV-1 IIIB, albeit in some cases less efficiently in comparison with CAP (Fig. 4). On the other hand, gp41 six-helix bundle formation in HIV-1 BaL was detectable only for CAP and poly(naphthalene sulfonate) treated virus (Fig. 5) suggesting differences among the anionic polymers with respect to their activity against the R5 virus HIV-1 BaL. The induction of six-helix bundles in HIV-1 does not seem to be the only mechanism involved in virucidal activity of anionic polymers (compare

Fig. 5 with Figs. 8 and 9) and there might be other contributing processes.

Since CAP in micronized form was shown to cause disintegration of HIV-1 virus particles [31], (Fig. 3), it was of interest to determine whether any of the other anionic polymeric microbicide candidates affect the integrity of virus particles. While HIV-1 IIIB was not affected significantly by any of the polymers tested, CAP to some extent, but surprisingly cellulose sulfate affected the integrity of HIV-1 BaL particles (Fig. 6). These results were supported by the observation that HIV-1 BaL gp120 after treatment with cellulose sulfate, unlike virus associated gp120, did not become precipitated with 3% PEG and failed to pellet by centrifugation under conditions leading to sedimentation of intact virus particles (Fig. 7)]. The differential sensitivity of an R5 virus, HIV-1 BaL, as compared to an R4 virus, HIV-1 IIIB, to the same negatively charged polyelectrolyte appears unprecedented to the best of our knowledge.

Among the three polysaccharide sulfate esters (carrageenan, cellulose sulfate and dextran sulfate), cellulose sulfate appeared to be more effective against HIV-1 BaL than the other polymers. This might be due to the polysaccharide backbone of cellulose [60] with a propensity to form strong intermolecular hydrogen bonds and to be involved in hydrophobic interactions more than soluble dextrans [61] and galactose + 3,6-anhydrogalactose copolymers (= the saccharide moiety of carrageenan) [62,63]. Among the two polymers containing aryl sulfonates, poly(napthalene sulfonate) was more effective than poly(styrene-4-sulfonate) with respect to induction of gp41 six-helix bundles in HIV-1 BaL. This difference may possibly be due to the high propensity of the uncharged part of the molecule {poly(naphtalene)} to be involved in hydrophobic interactions. Among the two carboxylate polymers (Carbomer 974P and CAP), only soluble CAP (at neutral pH) was able to elicit the formation of gp41 six-helix bundles in both HIV-1 IIIB and BaL and was virucidal against both of these viruses. The insoluble, micronized form of CAP had similar properties. The differences in biological activities of these polymers is probably due to their noncharged portions, i.e. mainly the occurrence of benzene rings in CAP.

Polycarboxylates (soluble CAP and Carbomer 974P; Fig. 10) had a minimal effect on plasma coagulation. Among the sulfonates and polysaccharide sulfates, carrageenan and poly(napthalene sulfonate) had lower anti-coagulant activity in comparison with cellulose sulfate, dextran sulfate and poly(styrene-4-sulfonate) (Fig. 10). Consideration of these side effects might contribute to the process of selection of preferred microbicides for future large-scale use.

Conclusions

Among anionic polymers considered as topical microbicides to prevent sexual transmission of HIV-1 {Carbomer 974P/BufferGel, carrageenan, CAP, cellulose sulfate, dextran/dextrin sulfate, poly(napthalene sulfonate) and poly(styrene-4-sulfonate)}, CAP formulated in a micronized form, is the only compound which has a capacity to remove from physiological fluids infectious virus by adsorption onto the micronized particles. The interaction between HIV-1 IIIB, a representative of X4 viruses, and HIV-1 BaL, a representative of R5 viruses, with the micronized form of CAP has the following consequences: 1) elicitation of "dead-end" gp41 six-helix bundles expected to render the virus incompetent to fuse with target cells; 2) loss of virus integrity and stripping off of the envelope glycoproteins; and 3) inactivation of virus infectivity. Elicitation of gp41 six-helix bundles and virucidal activity represent shared properties between micronized and soluble (at sufficient concentrations) forms of CAP [37].

As determined by ELISA, the aforementioned polymers other than CAP (except Carbomer 974P) also elicited gp41 six-helix bundle formation in HIV-1 IIIB, albeit less effectively than CAP. On the other hand, only poly(napthalene sulfonate), in addition to CAP, elicited detectable six-helix bundle formation in HIV-1 BaL. This suggests that X4 and R5 viruses may be differently affected by some of the polymeric compounds being considered as topical microbicides. This was confirmed by the results of assays for virucidal activity.

Abbreviations used

ACD, acid citrate dextrose; CAP, cellulose acetate phthalate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HIV-1, human immunodeficiency virus type 1; HRP, horseradish peroxidase; mAb, monoclonal antibodies; PEG 6000, polyethylene glycol 6000; PBS, phosphate buffered saline; PBS-BG, 1% BSA/1% gelatin in PBS; sCD4, soluble CD4; STD, sexually transmitted disease; TS, 0.14 M NaCl, 0.01 M Tris, pH 7.0;

Competing interests

None declared

Authors' contributions

Author 1 ARN developed the concepts representing the basis of the manuscript and designed most experiments. Author 2 NS carried out most experiments and contributed to the development of experimental techniques. Author 3 YYL did all the tissue culture work and infectivity assays.

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