Separation of Human Plasma Factor IX From HTLV-I or HIV by Immunoaffinity Chromatography Using Conformation-Specific Antibodies

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Immuoaffinity chromatography using conformation-specific antibodies yields pure factor IX from human plasma in a single rapid, facile purification step. We evaluated this technique to determine whether factor IX can be separated from human T cell leukemia virus-I (HTLV-I) and human immunodeficiency virus (HIV) in plasma supplemented with these viruses. Viral content was determined with an enzyme-linked immunosorbent (ELISA) assay sensitive to 50 ng viral protein. Both HTLV-I and HIV coeluted with unbound protein. Neither HTLV-I nor HIV was detected in purified factor IX. We conclude that, to the limits of detection, factor IX purified by this method is free of viral contamination.

MATERIALS AND METHODS

Preparation of HTLV-I and HIV. HTLV-I was produced from the cell line HUT 102 B2, and HIV was produced from the B cell line FCRC-15/AAV. Normal pooled human plasma was obtained from donors who were negative for HTLV-I and HIV antibody and antigen. Purified HTLV-I and HIV were prepared by centrifugation (45,000 × g) of cell-free conditioned media from the respective cell lines. Pelleted particles were sedimented with alternating discontinuous glycerol (30% over 100% cushion), continuous sucrose (22% to 65%), and discontinuous glycerol (30% over 100% cushion) gradients. Positive fractions were identified by assaying reverse transcriptase activity using the template primer poly (C) oligo dG in the presence of 10 mmol/L MgCl2.

Competitive enzyme-linked immunosorbent assay (ELISA) for HTLV-I and HIV. Polyclonal rabbit anti-HTLV-I and anti-HIV antibodies were raised in rabbits immunized with ether-disrupted purified virions, and the antibodies were measured by ELISA and Western blot assays for HTLV-I and HIV antibodies. Microtiter plates were coated with 200 ng viral protein (either HTLV-I or HIV) in 100 μL CBC (150 mmol/L Na2CO3; 350 mmol/L NaHCO3, pH 9.6) and then incubated overnight at 4°C. After incubation, excess solution was removed and 100 μL of 1% bovine serum albumin (BSA) in CBC was added to each well; the plate was incubated overnight at 4°C.

Dilutions of rabbit antisera (1/50 for HTLV-I and 1/5000 for HIV) were made in phosphate-buffered saline (PBS)-TWEEN 20. Competing proteins and diluted antisera were incubated together for 1 hour at 23°C. The viral protein-coated wells were washed three times with 0.1% BSA in PBS-TWEEN 20 and then 100 μL of the antisera mixture described above were added to the appropriate well. After incubation for 1 hour at 23°C, the plates were washed three times with PBS-TWEEN 20 and 100 μL of goat anti-rabbit IgG alkaline phosphatase conjugate (1/5,000 dilution in PBS-TWEEN 20) was added. The plates were incubated for 2 hours at 23°C, washed three times with PBS-TWEEN 20, and 100 μL of p-nitrophenyl phosphate was added to each well. The plates were developed for 30 minutes, and the reaction was stopped with 15 μL of 3 N NaOH. The plates were read at 405 nm.

Preparation of anti-factor IX: Mg(II) antibodies. Antibodies were isolated as previously described, except that a subpopulation of conformation-specific antibodies were used that bind to factor IX in the presence of Mg2+. Antibodies that bind to factor IX in the presence of Ca2+ but not Mg2+ were removed. Rabbit anti-factor IX: Mg(II) antibodies were coupled to cyanogen bromide-activated Sepharose 4B at a concentration of 2.5 mg/mL in 0.1 M Tris-HCl pH 7.4. In the experiment with HIV, factor IX was isolated on the anti-factor IX: Mg(II) antibody-Sepharose column. An anti-factor IX:Ca(II)–Sepharose column was used in the experiment with HTLV-I; this column has been previously described.

Isolation of Factor IX from virus-supplemented plasma with anti-factor IX: Mg(II) Sepharose. Pooled human plasma (100 mL) was dialyzed overnight against 0.05 mol/L Tris-HCl, pH 7.4, 0.5 mol/L NaCl. After dialysis, the plasma was adjusted to 7.5 mmol/L
with 1 mol/L MgCl₂. Then either 500 μg of HTLV-I or 500 μg of HIV was added to the plasma. As a control, an equal volume of plasma without added viruses was applied to the affinity column to assess nonspecific background in both the HTLV-I and the HIV assays. The column was developed as previously described except that Tween-20 was added to buffers only in the experiment in which HTLV-I was added to plasma. The flow-through was collected in 5.5-mL fractions initially and then 1.5-mL fractions during elution of factor IX with 10 mmol/L EDTA. The protein concentration was estimated by the absorption at 280 nm and by factor IX radioimmunoassay. Pooled fractions were subjected to high-speed centrifugation (49,000 g) for 60 minutes. The pellets were disrupted with ether and resuspended in 100 μL of PBS-Tween-20. Ten microliters were then assayed for HTLV-I or HIV.

RESULTS
Evaluation of viral content of affinity-purified factor IX. HTLV-I (500 μg) was added to pooled human plasma prior to isolating factor IX on the anti-factor IX:Ca(II)-Sepharose column. HTLV-I coeluted with unbound protein. No HTLV-I was detected in the material that was eluted from the column with either EDTA or guanidine HCl (Fig 1). By this analysis, a minimum of a 1,000-fold reduction in HTLV-I occurred in face of a 10,000-fold increase in the specific activity of factor IX.

In a separate experiment, HIV (500 μg) was added to pooled plasma prior to the application of the plasma to the anti-factor IX:Mg(II)-Sepharose column. The HIV coeluted with unbound protein. No viral contamination was detected in the material eluted from the column with either EDTA or guanidine HCl (Fig 2). As previously, a minimum of a 1,000-fold reduction in HIV occurred in face of a 10,000-fold increase in the specific activity of factor IX.

The standard curves for the competitive ELISA demonstrated a sensitivity to 50 ng viral protein (Fig 3). Pooled human plasma without endogenous or added viral contaminants was applied to the anti-factor IX:Mg(II)-Sepharose column. The unbound and bound fractions obtained from this experiment were assayed for HTLV-I and HIV to determine accurately the level of nonspecific binding in the assay. The OD₅₀ values observed were equivalent to the OD₅₀ values observed in the factor IX prepared from experimentally contaminated plasma samples.

DISCUSSION
This study examined the behavior of two of the human retroviruses, HTLV-I and HIV, when they are present in plasma used for purification of factor IX. Our data indicate that, to the limits of detection of the immunoassay systems used, these viruses do not copurify with factor IX. The virus...
and the factor IX that is eluted with EDTA are resolved. Our data from these and earlier experiments indicate a general method for the separation of factor IX from viruses using an immunoaffinity purification involving specific binding and specific elution.

The morbidity associated with infusion of currently available factor IX concentrates has been well described. Recent epidemiologic data on the development of the acquired immunodeficiency syndrome in patients with hemophilia are of great concern. Thirty percent to 36% of the patients treated with factor IX concentrate showed HIV antibody positivity. 

Lyophilization of factor IX concentrates results in a 10- to 100-fold decrease in virus. Heat treatment of factor IX concentrates for 20 hours at 60°C renders HIV undetectable. Assumptions regarding the complete sterilization of factor IX concentrates from HIV contamination are done by extrapolation of the data derived from immunoassays. A small group of hemophilia A patients whose sera were initially negative for anti-HIV antibodies have received heat-treated factor VIII concentrate for up to 1 year without seroconverting. This suggests marked improvement can be anticipated in treatment of hemophilia B patients with heat-treated factor IX concentrate. Heat treatment, however, results in the loss of ~8% to 40% of the factor IX coagulant activity of the factor IX concentrate.

Our technique provides a rapid, one-step quantitative purification of homogeneous factor IX. We showed that, to the limits of detection, the factor IX isolated by this method is free of retrovirus, even when these viruses are added in high titer to plasma. These results indicate that this immunoaffinity method might be integrated into methods of manufacture of factor IX used for the treatment of hemophilia B. Whether this method can eliminate the need for heat treatment of plasma to eliminate HIV or render the factor IX free of hepatitis viruses will require direct infectivity assays or systematic study of patients treated with this new preparation.

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SA Limentani, BC Furie, BJ Poiesz, R Montagna, K Wells and B Furie