Factor VIII:C Concentrate Purified From Plasma Using Monoclonal Antibodies: Human Studies

By D.B. Brettler, A.D. Forsberg, P.H. Levine, J. Petillo, K. Lamon, and J.L. Sullivan

Conventional clotting factor concentrates have, until recently, been "of intermediate purity," containing <1% of the coagulation factor, and >99% extraneous plasma proteins such as fibrinogen, fibronectin, gamma globulins, and traces of many others. We report here the results of a new factor VIII concentrate that is purified from human plasma using a mouse monoclonal antibody to factor VIII:vWF in an affinity chromatography system. The resultant concentrate has an activity of between 3,000 and 5,000 U/mg protein before albumin is added as a stabilizer. Seven patients with severe hemophilia A and no inhibitor who were positive for antibody to human immunodeficiency virus (HIV) have been treated solely with this concentrate for over 24 months. Factor usage in these patients has ranged from 611 U/kg/yr to 2,022 U/kg/yr. These patients have infused approximately once per week on the average, most often for joint hemorrhages. The efficacy of the concentrate is excellent. No allergic reactions have occurred and no factor VIII antibodies have developed. In these seven patients mean CD4 counts stabilized (856 ± 619 at screen v 778 ± 686 at 24 months) and there was reversal of skin test anergy. In a comparison group on conventional intermediate purity concentrate chosen retrospectively decreases in mean CD4 counts similarly did not occur. However, the number of the comparison patients who were anergic increased over the course of the study. These observations indicate the possibility that more highly purified concentrates may stabilize immune function in HIV seropositive patients.

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seven hemophiliacs on conventional intermediate purity factor VIII concentrate that was dry heat treated (six patients) or organic solvent "wet" treated (one patient). This group was matched with the above patients for HIV serologic status, age, number of CD4 lymphocytes at the start of the study, and annual factor usage. The median age was 28 (range, 15 to 33 years) and median factor usage was 1,082 U/kg/yr (range, 722 to 3,232). Characteristics of this comparison group are seen in Table 1.

Methods

Factor VIII concentrate (Monoclate; Armour Pharmaceuticals, Blue Bell, PA). Cryoprecipitate, the source material, was passed over an agarose gel column to which a mouse monoclonal antibody to factor VIII:C was then covalently bound. Factor VIII:C was then eluted from the column using a calcium chloride gradient, concentrated, and further purified on a second affinity column. The resultant concentrate contained 700 to 3,500 units factor VIII:C/mg protein, which decreased in the final preparation to 5 units of factor VIII:C/mg protein after albumin was added as a stabilizer. vWF was present in small quantities and murine monoclonal antibody was present in minute amounts (from undetectable to 50 ng/mL in the final product). To enhance viral safety, after the addition of albumin the concentration was lyophilized and then heat treated to 60°C for 30 hours. By Western blot analysis, the factor VIII molecule retains the same molecular structure as the factor VIII concentrate that is prepared by the conventional method. There is a >5 log removal of added HIV with affinity chromatography plus heat treatment. Other model viruses including sindbis virus, vesicular stomatitis virus, and pseudorabies virus are also significantly reduced during the purification process and rendered undetectable by heat treatment. The concentrate reconstitutes within seconds into a clear solution.

Factor VIII half-life and recovery. Each patient had a half-life and recovery study at entry into the protocol. The patient was given approximately 25 U/kg of factor VIII concentrate. Blood was drawn at 30 minutes and at 1, 2, 4, 6, 8, 24, and 48 hours after the infusion; factor VIII levels were determined at each time point. Using linear least squares, a linear equation was estimated for the terminal phase of the factor VIII activity level against time curve. In vivo half-life (t½) was then estimated from the formula \( t_{1/2} = \frac{0.693}{B} \), where B = slope of factor VIII activity vs time profile. Recovery (K) in units per deciliter was calculated by the following formula: K = body weight (kg) x factor VIII rise (U/dL)/dose of factor VIII concentrate administered (u). Half-lives and recovery data were also obtained at 6 months, as a sensitive measure of possible development of low level inhibitor antibody. Inhibitor assays were carried out at screen, and at 6, 12, and 24 months, using the Bethesda method of Kasper et al.7

Immunologic testing. Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc, Piscataway, NJ) density gradients. Relative percentages of T-lymphocyte populations were enumerated using indirect immunofluorescence and the following mouse monoclonal antibodies: OKT1, OKT4, and OKT8 (Ortho Pharmaceutical Co, Raritan, NJ). All samples were analyzed using a Becton-Dickinson FACS IV fluorescence activated cell sorter. Absolute numbers of various cell populations per microliter of whole blood were calculated by multiplying their relative percentage by absolute numbers of mononuclear cells as determined from complete blood counts.

Delayed cutaneous hypersensitivity. The Multitest skin test antigen set (Multitest CMI; Merieux Institute, Inc, Miami, FL) was used on each patient, as reported previously. The seven antigens included tetanus toxoid, candida, proteus, tuberculin, diphtheria toxoid, staphylococcus, trichophyton, and a glycerol control. The test was applied to the volar surface of the forearm and read at 48 hours by the patient using a guide provided by the center after a teaching program. A response was considered positive if the induration measured 2 mm or greater. A patient was considered anergic if he did not respond to any of the seven test antigens. A patient was considered nonanergic if he responded to one or more test antigens.

Measurement of human anti-murine IgG. Human anti-murine IgG was measured by an ELISA technique. Flexible 96 well polivinylchloride plates (Dynatech) containing 50 µL/well of murine monoclonal antibody used during the factor VIII:C purification process or a human IgG standard suspended in PBS were incubated at 37°C. The plates were then aspirated and washed, after which 50 µL of patient serum or control were placed in the wells, incubated for four hours, at 4°C, and washed three times with PBS buffer. Fifty microliters 125I goat anti-human IgG was placed in each well; the plates were incubated for 15 hours at 4°C, washed three times with buffer, and the individual wells counted in a gamma counter. Absolute counts per minute (cpm) values were computed into bound over total input cpm percentages. A standard curve was constructed from the microtiter wells coated with 50 µL human IgG ranging in concentration from 0.5 to 20 µg/mL.

Other testing. Other testing included response of lymphocytes to the lectin concanavalin A (Con A), circulating immune complexes, and IgE levels. Response to Con A was measured using a standard microculture technique where 5 x 10⁶ PBMC in 0.25 mL of RPMI 1640 (Grand Island Biological Co, Grand Island, NY) supplemented with 10% fetal calf serum (Grand Island Biological Co) were distributed into replicate wells of Microtiter II plates (Falco Labware, Oxnard, CA). Con A (Calbiochem-Bohring Corp, San Diego) was added in concentrations of 0.4 to 40 µg/mL and the plates cultured for five days at 37°C in a 5% CO₂ environment. Eighteen hours before harvesting, the cells were pulsed with 3H thymidine, (specific activity, 6.7 µCi/mmol; New England Nuclear, Boston). Cells were harvested onto glass fiber filters and 3H thymidine content was determined by scintillation counting. All cultures were performed in triplicate. Results are reported as maximum cpm.

Circulating immune complexes were measured at screen and at 6 months using the Raji method. Normal values range between 0 and 50 µg aggregated human gammaglobulin (AHG) equivalents per milliliter. IgE levels were determined using a simultaneous sandwich technique (Kallestad, Austin, TX). Briefly, to a 20 µL serum sample, a mixture of 125I-radiolabeled equine anti-human IgE and cold mouse anti-human IgE were added and incubated. Goat anti-mouse IgG was then added, the mixture incubated, and the tube
centrifuged to separate the bound radiolabeled antibody-IgE complexes that precipitated. The supernatant was decanted and the precipitate counted to measure the bound radioactivity. The amount of radioactivity was proportional to the concentration of IgE in the sample as determined by a calibration curve. Results were considered normal in the range of 0 to 180 IU/mL.

*p24 Antigen.* Serum samples were analyzed for p24 antigen using the Abbott HTLV III EIA Kit (#4234-22 Abbott Diagnostics, Chicago). Briefly the assay is a "sandwich" solid phase enzyme immunoassay. The sample is considered positive or negative for HIV antigen(s) by comparing the absorbance at 492 nm of the specimen to a cutoff value. The cutoff value is the absorbance of the mean of the negative control plus the factor of 0.050. Samples with absorbance values greater than or equal to the cutoff value are considered reactive for HIV antigen(s).

Statistical analysis. The Paired Student's t test was used to compare the means of lymphocyte cell counts at screen and 24 months of the group on experimental factor or conventional factor and also to compare the means of the group on experimental factor concentrate vs conventional factor concentrate at the 24-month period.

**RESULTS**

**Safety and Efficacy**

The median amount of factor concentrate used by each patient during the study is shown in Table 1. Infusions were given for joint and soft tissue hemorrhages, and in one case for arthroscopy. Hemostatic efficacy was judged excellent by all patients for all episodes. When the seven patients were infused under physician supervision, there were no changes in vital signs. No side effects of the factor were noted during infusion under physician supervision, there were no changes in vital signs. No side effects of the factor were noted during

**Immunologic Results**

As seen in Table 3, at screen the mean CD4 cell level was 856 ± 619 cells/μL and at 24 months it was 778 ± 686 cells/μL, which was not a significant change. The total amount of T cells was 1,206 ± 529 cells/μL at screen and 1,789 ± 1,888 cells/μL at 24 months; helper/suppressor cell ratios decreased slightly but not significantly over the 24 months of the trial (from 1.11 ± 0.34 to 0.91 ± 0.52). Skin test anergy decreased in the group on experimental factor as seen in Table 4. The number of patients that were anergic at the onset of the study (three of seven) decreased to one of seven at the 24 month point. At screen, the mean number of skin test antigens to which the group responded was 1.6 ± 1.6. At 24 months, the mean number of skin test antigens to which the patients responded was to 2.9 ± 1.7 antigens, a nonsignificant difference.

When immunologic function was compared over 24 months to the subset of seven matched patients that were on conventional concentrate, it was found that the group on conventional concentrate also did not have a significant decrease in mean CD4 cell level (684 ± 282 cells/μL v 603 ± 403, P = NS) over 2 years. (Table 5). However, while only one subject in the experimental group (patient no. 7) dropped his CD4 count by 50% or more, three of the comparison subjects (patients 4C, 6C, and 7C) have developed AIDS while two patients on the conventional concentrate have become ill with AIDS. Of interest also is that of seven patients tested for p24 antigen in the experimental group one was positive (patient no. 7), while no patient of six tested in the comparison group was positive (data not shown). Patient no. 7, even with decreases in CD4 cell count and a positive p24 Ag remains asymptomatic. Additionally, liver function tests

![Fig 1. Representative factor VIII half-life at screen (first dose) and at 6 months (second dose).](image-url)
were not significantly different between the experimental group and comparison group at the outset of the study and this did not change during the 2-year course of study (data not shown).

Additional Immune Studies

The results of con A stimulation showed no significant change in the experimental group between screen values (48,146 ± 24,646 cpm) and values at 18 months (32,831 ± 16,554 cpm). Levels of circulating anti-mouse antibody were in the lower limits of the assay (5 to 7.8 μg/mL) before the start of the study for six of seven patients. One patient had an elevated screen level of 13.8 μg/mL due to the presence of rheumatoid factor, which interfered with the ELISA assay. No increase in levels was seen over 6 months of the study except for the patient with the rheumatoid factor whose level rose to 25.2 μg/mL. Mean IgE level was 131.1 ± 91.1 IU/mL (normal range, 0 to 180) at screen and at 6 months mean levels were 115.9 ± 88.3. Mean circulating immune complexes were elevated at screen (360 ± 73 μg AHG eq/mL; range, 243 to 462) and did not change significantly when tested at 6 months (366 ± 167; range, 246 to 718). Subsequent testing for IgE, circulating immune complexes, and anti-mouse IgG was not repeated. No patient developed an inhibitor to factor VIII over the course of the study.

DISCUSSION

Factor concentrate purified using affinity chromatography and a monoclonal antibody to factor VIII:vWF has been shown by this study to be safe and efficacious over a prolonged period of time. It has been demonstrated by others that activating latently HIV infected mononuclear cells with mitogens may lead to HIV replication. More recently it has been demonstrated that latently infected cells may also be stimulated to express HIV when stimulated by other viruses such as CMV and hepatitis B. Because >80% of patients with severe hemophilia over the age of 5 years are positive for HIV antibody and thus presumably latently infected with HIV, the theoretic advantage of purer factor VIII concentrate product may be less expression of HIV in an infected patient over a period of time.

In the seven patients studied, stabilization of CD4 cell levels seemed to occur, as well as an improvement in delayed cell mediated immunity as determined by decrease in the number of patients who demonstrated skin test anergy. However, in the comparison group, matched for HIV sero-positivity, CD4, level, age, and factor usage, there was also no significant decrease in mean CD4 levels over 2 years. The number of patients that were anergic at the outset decreased over 2 years in the group using experimental concentrate, while the number of patients that were anergic increased in the group on conventional concentrate. Within each group, however, the mean number of individual antigens to which the patients responded to did not change. The numbers of positive responses also was not significantly different between the two groups at the end of 24 months. Two patients have developed AIDS in the group using conventional intermediate purity concentrate while all patients remain asymptomatic on the experimental factor. The number of patients, however, is very small. Additionally, the comparison group was chosen retrospectively. Thus, definitive conclusions about effects of the experimental concentrate on immune system stabilization or improvement cannot be drawn.

It is interesting to note that in in vitro experiments looking at the effect of factor concentrates on human mixed lympho-

Table 3. Quantitative CD4 Cell Subsets (Cells/μL) in Patients on Experimental Concentrate

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<th>Patient</th>
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<th>10</th>
<th>14</th>
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<td>Mean ± SD</td>
<td>856 ± 619</td>
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<td>810 ± 620</td>
<td>922 ± 620</td>
<td>750 ± 573</td>
<td>705 ± 434</td>
<td>778 ± 686</td>
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Table 4. Number of Skin Test Antigens to Which Each Patient on Experimental Concentrate Responded

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<td>4</td>
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<tr>
<td>Mean ± SD</td>
<td>1.6 ± 1.6</td>
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<td>2.9 ± 1.9</td>
<td>3.5 ± 1.5</td>
<td>2.7 ± 0.8</td>
<td>2.9 ± 1.7</td>
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Abbreviations: ND, not done; NE, not evaluable.
cyte reactions and phytohemagglutinin mitogenesis, conventional concentrates induced 50% inhibition at approximately 1 mg/mL protein concentration, while the experimental concentrate induced no inhibition at any concentration examined. Additionally, intermediate purity concentrates now have been shown to downregulate Fc receptors on monocytes. This data may have clinical relevance in determining if “purer” factor VIII concentrate may improve or stabilize deteriorating immune function.

Whether HIV and non-A, non-B hepatitis virus are completely eliminated by this new manufacturing process cannot be assessed by this study since all the patients studied were HIV antibody positive at the outset and also had presumed non-A, non-B hepatitis secondary to previous long-term exposure to conventional concentrates. This issue is currently being addressed in a study involving approximately 40 hemophiliac patients who had been previously untransfused and now have been using only the highly purified concentrate. Additionally, now that adequate supplies of this new blood derivative are available, a randomized controlled study of a large number of patients is ongoing in order to attempt to determine conclusively whether purer factor concentrates are of benefit for persons with hemophilia who have previously been infected with HIV. Only when such randomized trials are completed can the issue of cost/benefit of these new more expensive concentrates be adequately answered.

REFERENCES

Factor VIII:C concentrate purified from plasma using monoclonal antibodies: human studies

DB Brettler, AD Forsberg, PH Levine, J Petillo, K Lamon and JL Sullivan