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

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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 vs 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 occurred. They 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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Other selection criteria included: (a) no evidence of AIDS or of AIDS-related complex; (b) normal platelet count, (c) residence in proximity to the center to expedite frequent visits, and (d) willingness to sign informed consent. Characteristics of the seven patients are presented in Table 1.

The study participants initially underwent a recovery and half-life study where approximately 22 to 32 U/kg factor VIII were infused and blood was drawn after measured intervals as detailed below. Vital signs including blood pressure, pulse, respiratory rate, and temperature were monitored. Subsequently two factor concentrate infusions were administered in the clinic setting, observed, and monitored by the center staff. After these infusions, the patients used the concentrate on a home care basis as described previously. They infused on the average of 15 to 25 U/kg/wk with a total dosage median of 1,454 U/kg/yr (range, 611 to 2,022). One patient was additionally infused for a minor surgical procedure and the same patient was infused for trauma secondary to an automobile accident.

The patients were observed closely with periodic physical examinations and laboratory testing. Laboratory testing included immunologic parameters such as CD4, CD8 cell counts, concanavalin A lymphocyte stimulation, and delayed cutaneous hypersensitivity as measured by skin testing as detailed in the next section. These were performed every 3 to 6 months for 24 months. Additionally, IgE levels and circulating immune complexes were drawn at screen and 6 months, factor VIII inhibitor titers were obtained every 6 months, antibody levels to mouse IgG were measured every 4 months, and half-life and recovery studies were obtained at screen and 6 months. In order to assess whether changes noted in immune function in the seven patients during the study were significant, a comparison group was retrospectively chosen. It consisted of a matched group of

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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, P.A.).** Cryoprecipitate, the source material, was passed over an agarose gel column to which a mouse monoclonal antibody to factor VIII:vWF (made by Dr T. Zimmerman, Scripps Clinic, La Jolla, CA) was 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 its immunologic and biologic activity. It contains no detectable contamination with viruses, streptococcus, trichophyton, and a glycerol control. The test was applied to the volar service 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 polyclonalplate controls (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 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 10⁴ 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 (Falcon Labware, Oxnard, CA). Con A (Calbiochem-Boerhing 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/mm; 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 screening 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 ³H-labeled 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 Enzyme Immunoassay. 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 0.05. Samples with absorbance values greater than or equal to the cutoff value are considered reactive for HIV antigen(s).

**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 the study. Recovery was excellent and was not significantly different when determined at screen and at 6 months into the study. At screen the recovery value was 1.9 ± 0.3 U/dL/kg and at 6 months it was 2.2 ± 0.4 U/dL/kg; the anticipated recovery for factor VIII is 2.0 U/dL/kg. The half-life was also comparable with conventional concentrates of 15.4 hours ± 2.2 at screen and 17.5 hours ± 5.9 at 6 months (expected, 8 to 12 hours). The half-life and recovery data are seen in Table 2. A representative t½ curve is seen in Fig. 1.

**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 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) had declines of this magnitude. One patient on conventional concentrate was anergic at the outset, as compared with three patients on the experimental concentrate. After 24 months two of six patients on conventional concentrate were anergic as compared with one of seven patients on the experimental factor.

Clinically, no patient on the experimental factor has developed AIDS while two patients on the conventional intermediate purity concentrate (6C, 7C) have become ill with AIDS. Of interest also is that of six 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

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**Table 2. Half-Life and Recovery of Monoclonally Purified Factor VIII**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Weight (kg)</th>
<th>Dose (U)</th>
<th>Maximum FVIII Level</th>
<th>Half-Life (hr)</th>
<th>Recovery</th>
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<tbody>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>5.9</td>
<td>0.35</td>
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</table>

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![Fig 1. Representative factor VIII half-life at screen (first dose) and at 6 months (second dose).](#)
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 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>
<thead>
<tr>
<th>Patient</th>
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<th>14</th>
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<td>195</td>
<td>ND</td>
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<td>131</td>
<td>38</td>
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</tbody>
</table>

Mean ± SD: 856 ± 619, 802 ± 651, 810 ± 620, 922 ± 620, 750 ± 573, 705 ± 434, 778 ± 686

Table 4. Number of Skin Test Antigens to Which Each Patient on Experimental Concentrate Responded

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 antihemophilic factor concentrate precipitate.

Deteriorating immune function. Additionally, intermediate purity concentrates 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