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

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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 cell 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.

FACTOR VIII DEFICIENCY (hemophilia A) has historically been treated with a variety of human blood products. Until the 1960s, whole blood and then fresh frozen plasma were used. In 1964, Pool et al discovered that factor VIII could be partially purified by precipitation in the cold.[1] Cryoprecipitate was subsequently adopted as the accepted treatment of hemophilia A. Lyophilized factor VIII concentrate was introduced in the late 1960s.[2] It has been the therapy of choice since the early 1970s, enabling hemophiliacs to treat themselves in a home therapy setting and thus allowing the patients to greatly normalize their life functions.[3] Factor VIII concentrate is made from large plasma pools and has been associated in the past with transmission of hepatitis B, non-A, non-B hepatitis (NANBH), delta hepatitis, parvovirus, and human immunodeficiency virus (HIV).[2,4] Since 1984, viral inactivation processes such as heat treatment during preparation of concentrates have become widely available commercially. Currently all factor concentrate used in the United States has undergone viral inactivation.

It has been postulated that intermediate purity factor concentrates could directly or indirectly cause immunosuppression because of the many foreign proteins and alloantigens that they contain.[5,6] Thus, a more highly purified concentrate might be advantageous because it would replace primarily the missing clotting factor, reducing the infusion of extraneous plasma proteins and of living or dead viruses. We report here the results of the use of factor VIII concentrate purified using affinity chromatography with murine monoclonal antibody to human von Willebrand factor (vWF) in the first seven human patients to receive this material. These patients all had severe hemophilia A and all had been exposed to HIV in the past. All seven have been observed for 24 months on this treatment.

PATIENTS AND METHODS

Patients

Seven patients observed at the New England Area Comprehensive Hemophilia Center who had severe factor VIII deficiency (factor VIII<1%) were selected. They ranged in age from 16 to 39 years (median, 26 years). All were HIV antibody positive since 1983, and all had antibody to hepatitis B surface antigen (HBsAb positive). 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.[3] 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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Table 1. Demographic Information

Patient	Hemophilia Type	Age	Dose (U/kg/yr)
Experimental group			
1	A Severe	27	829
2	A Severe	28	611
3	A Severe	26	770
4	A Severe	25	1,559
5	A Severe	22	2,002
6	A Severe	16	2,022
7	A Severe	39	1,454
Comparison group			
1C	A Severe	28	1,082
2C	A Severe	30	782
3C	A Severe	23	722
4C	A Severe	28	2,071
5C	A Severe	24	863
6C	A Severe	15	1,359
7C	A Severe	33	2,232

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: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 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 alone and a >9.1 log removal of HIV after affinity chromatography plus heat treatment.[7] Other model viruses including sindbis virus, vescicular stomatitis virus, and pseudorabies virus are also significantly reduced during the purification process and rendered undetectable by heat treatment.[7] 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 ($t^1/_2$) was then estimated from the formula $t^1/_2 = .693/B$, where B = slope of factor VIII activity *v* time profile. Recovery (K) in units per deciliter was calculated by the following formula: K = body weight (kg) × 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.[8]

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: OKT11, OKT4, and OKT8 (Ortho Pharmaceutical Co, Raritan, NJ). All samples were analyzed using a Becton-Dickenson FACS IV fluorescence activited 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.[9] The seven antigens included tetanus toxin, candida, proteus, tuberculin, diptheria toxoid, 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.[7] Flexible 96 well polvinylchloride 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 ^{125}I 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[10] where 5×10^4 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-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% CO_2 environment. Eighteen hours before harvesting, the cells were pulsed with ^{3}H thymidine, (specific activity, 6.7 μCi/mmol; New England Nuclear, Boston). Cells were harvested onto glass fiber filters and ^{3}H 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.[11] 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 ^{125}I-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 Antigen 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 *v* 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 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 $t^1/_2$ 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 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 CD_4 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 CD_4 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

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

Patient	Weight (kg)	Dose (U)	Maximum FVIII Level	Half-Life (hr)	Recovery
Screen/baseline					
1	74.6	2,135	48	15.4	1.7
2	83.2	2,562	63	13.7	2.0
3	70.5	2,135	49	13.4	1.6
4	110.0	3,416	64	12.6	2.1
5	65.9	2,135	52	18.4	1.6
6	60.9	1,640	47	17.5	1.7
7	65.5	1,640	61	16.6	2.3
Mean				15.4	1.9
SD				2.2	0.27
Six months					
1	75.4	1,840	55	14.4	2.3
2	82.7	2,080	72	11.0	2.9
3	70.9	1,840	53	16.3	2.0
4	113.6	2,800	50	14.4	1.9
5	72.3	1,600	45	29.1	2.0
6	60.4	1,440	48	20.5	2.0
7	65.5	1,710	64	16.6	2.4
Mean				17.5	2.2
SD				5.9	0.35

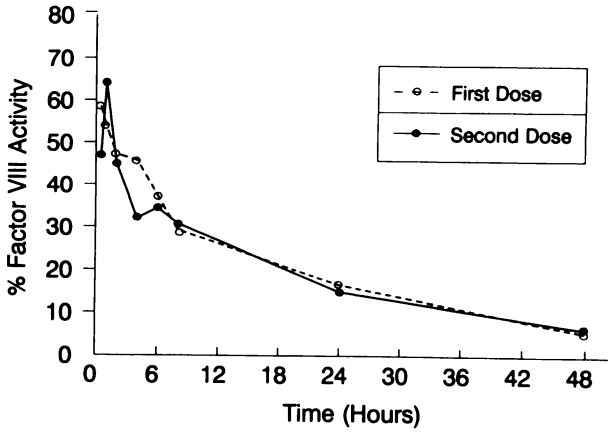

Fig 1. Representative factor VIII half-life at screen (first dose) and at 6 months (second dose).

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

		Months					
Patient	Screen	3	6	10	14	18	24
1	627	991	660	864	727	485	581
2	1,219	707	943	868	777	620	1,280
3	899	677	561	810	813	813	707
4	2,058	1,980	2,125	2,116	1,930	1,500	2,071
5	535	167	607	482	494	920	358
6	356	ND	576	392	369	469	414
7	297	288	195	ND	145	131	38
Mean ± SD	856 ± 619	802 ± 651	810 ± 620	922 ± 620	750 ± 573	705 ± 434	778 ± 686

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.[12] 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.[13,14] 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 seropositivity, CD_4 level, age, and factor usage, there was also no significant decrease in mean CD_4 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 4. Number of Skin Test Antigens to Which Each Patient on Experimental Concentrate Responded

		Months				
Patient	Screen	3	6	14	18	24
1	0	0	2	1	2	4
2	0	ND	2	5	4	4
3	3	2	3	3	3	4
4	2	5	3	3	NE	3
5	4	4	6	5	3	4
6	0	1	4	ND	2	1
7	2	3	0	4	2	0
Mean ± SD	1.6 ± 1.6	2.5 ± 1.9	2.9 ± 1.9	3.5 ± 1.5	2.7 ± 0.8	2.9 ± 1.7

Abbreviations: ND, not done; NE, not evaluable.

Table 5. Results of Immune Studies in Comparison Group

Patient	CD4 (cells/μL)*	CD4 (cells/μL)†	Skin Test* (No. of Antigens Positive)	Skin Test† (No. of Antigens Positive)
1C	743	874	ND	2
2C	1,037	692	2	4
3C	760	895	2	2
4C	996	445	0	ND
5C	563	882	1	2
6C	297	22	0	0
7C	397	132	1	0
Mean ± SD	684 ± 282	603 ± 403	1.8 ± 1.5	1.7 ± 1.9

Abbreviation: ND, not done.

*Represents values at screen.

†Represents values at 2 years.

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.[15] Additionally, intermediate purity concentrates have been shown to downregulate Fc receptors on monocytes.[16] 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 hemophilic 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.

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