A Component of Factor VIII Preparations Which Can Be Separated From Factor VIII Activity Down Modulates Human Monocyte Functions

By Martha M. Eibl, Rafi Ahmad, Hermann M. Wolf, Yendra Linnau, Elisabeth Götz, and Josef W. Mannhalter

In this study we investigated different aspects of monocyte functions following interaction of monocytes (Mo) with therapeutic concentrates of factor VIII (F VIII) concentrate. A short (one-hour) treatment of normal Mo with F VIII concentrates led to a significant \( P < 0.001 \) down modulation of Fc receptors expressed in the Mo plasma membrane. This down modulation was accompanied by a decrease of Mo effector functions that was expressed by a reduced capacity of F VIII-treated Mo to release \( {O}_2 \) radicals (40% of controls) and to kill bacteria (% killing: control Mo, 65%; F VIII-treated Mo, 24% to 51%). Further studies showed that the modulating activity was due to a contaminant present in F VIII concentrates (immune complexes or IgG aggregates). Fractionation using molecular sieving revealed that the modulatory activity was confined to a high-molecular range fraction (Mr > 1,270,000 daltons), while the fraction containing monomeric IgG had no effect. Further fractionation by affinity chromatography on protein A-Sepharose separated the coagulation activity (eluent) from the Mo function-modulating activity (eluate). We conclude that treatment with F VIII concentrates might contribute to an immunocompromised state in some hemophiliacs and facilitate opportunistic infections in these patients.

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MATERIALS AND METHODS

Factor VIII concentrates. Lyophilized factor VIII (F VIII) concentrates (Kroybulin M, lot #09M09083, Immuno AG, Vienna, Austria) were dissolved according to the instructions on the enclosed package insert (10 mL distilled water per 250 IU F VIII) and adjusted to the appropriate concentration in RPMI 1640 medium (Flow Laboratories, Irvine, England) supplemented with L-glutamine (2 mmol/L) and antibiotics (100 IU/mL penicillin, 100 \( \mu \)g/mL streptomycin). Further definition of the F VIII concentrate used is given in Table 1.

Fractionation of F VIII concentrates. Fractionation of F VIII concentrates was first performed according to molecular size using gel chromatography. A Biogel A-1.5 M (Bio-Rad Laboratories, Richmond, CA) column (100 to 200 mesh, column size 2.6 x 95 cm) was loaded with 5 mL (89.2 IU) of F VIII and eluted at a flow rate of 27 mL/hour (elution buffer 0.024 mol/L sodium citrate, 0.12 mol/L sodium chloride, pH 7.2). This fractionation yielded five peaks with virtually all the F VIII activity confined to peak 1. The protein-containing fractions from peaks 1 to 5 were pooled, transferred into dialysis tubings (20 DM-X100 FT, Union Carbide, Chicago), and concentrated by embedding into polyethylene glycol 20,000 (Serva, Heidelberg, FRG) to an F VIII activity of 2 IU/mL in fraction 1. The volumes of fractions 2 to 5 were reduced to the same extent. The protein content of each fraction is shown in Table 2.

A part of fraction 1 (Mr > 1,270,000) was further subjected to affinity chromatography on protein A-Sepharose (protein A-Sepharose CL-4B, Pharmacia, Uppsala, Sweden; column size 1.6 x 4.5 cm, starting buffer 0.024 mol/L sodium citrate, 0.12 mol/L sodium chloride, pH 7.2; elution buffer 0.05 mol/L sodium citrate, 0.05 mol/L citric acid, pH 3.0; flow rate 28 mL/hour). The fractions eluting from the protein A-Sepharose column were immediately neutralized using 1 mol/L NaOH. The protein-containing fractions from both the protein A-Sepharose eluent and eluate were pooled and concentrated as described above.

Prior to use in cell culture experiments, all F VIII fractions were extensively dialyzed against Dulbecco's phosphate-buffered saline (PBS) without Ca\(^{2+}\) and Mg\(^{2+}\). For monocyte pretreatment studies, Ca\(^{2+}\) and Mg\(^{2+}\) ions were added immediately before the incubation step.

Poly IgG. Human serum IgG (Cohn fraction II, a kind gift from Immuno AG) was dissolved at a concentration of 10 mg/mL in Dulbecco's PBS containing Ca\(^{2+}\) and Mg\(^{2+}\) ions (Flow Laboratories) and polymerized by heat aggregation at 63°C for 20 minutes. To remove large aggregates the preparation was then centrifuged at 600 x g for ten minutes.

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Isolation of mononuclear cells and preparation of monocyte monolayers. Mononuclear cells (MNC) were isolated from the peripheral blood of healthy human volunteers by buoyant density gradient centrifugation.\textsuperscript{11} Anticoagulated blood (40 IU preservative-free heparin (Immuno AG, Vienna, Austria) per mL) was layered on Lymphoprep (Nyegaard & Co, Oslo, Norway) and centrifuged at 400 x g for 35 minutes. Then the mononuclear cells from the interphase were aspirated, washed three times in 0.15 mol/L NaCl, and suspended in RPMI 1640 supplemented with 15% pooled, heat-inactivated (30\(^\circ\)C) human AB serum, penicillin (100 IU/mL), streptomycin (100 \(\mu\)g/mL), and L-glutamine (2 mmol/L) (complete medium).

Monocytes (Mo) were prepared by adherence to plastic surfaces. MNC were suspended in complete medium at a concentration of 1 x 10\(^7\) cells/mL. Two-milliliter portions thereof were pipetted into plastic tissue culture plates (Macro Tray, KremsmOnster, Austria) and incubated at 37\(^\circ\)C in a CO\(_2\) incubator for 16 hours. Adherent cells prepared in this way contained more than 90\% Mo, as judged by nonspecific esterase staining.\textsuperscript{14}

Pretreatment of Mo monolayers. Mo pretreatment was performed by incubating Mo monolayers with F VIII, F VIII fractions, or polymeric IgG for one hour at 37\(^\circ\)C immediately after adherence. The F VIII concentrates were used at concentrations of 2 IU/mL, 5 IU/mL, and 10 IU/mL; protein concentrations of the respective F VIII fractions are shown in Table 2; polymeric IgG was applied at a protein concentration of 10 mg/mL. Mo pretreatment with buffer solutions (RPMI 1640 supplemented with L-glutamine and antibiot-ics and/or PBS containing Ca\(^{2+}\) and Mg\(^{2+}\) ions) was always included as a control. At the end of the 1-hour incubation step, the substances used for pretreatment were aspirated, and the Mo monolayers were washed with 0.15 mol/L NaCl and kept in complete medium at 37\(^\circ\)C (CO\(_2\) incubator) for 16 hours.

Determination of Fc receptors. Receptors for the Fc portion of IgG on the Mo membrane were determined by rosette formation with antibody-coated ox red blood cells.\textsuperscript{13} Ox red blood cells were first sensitized with IgG by incubating equal volumes of ox red blood cells (2\% suspension in PBS) and rabbit anti-ox red blood cell antibodies (IgG fraction, 0.167 mg/mL PBS, Nordic Immunology, Tilburg, The Netherlands) at 37\(^\circ\)C for one hour.\textsuperscript{14} Mo monolayers were washed twice with PBS containing 0.2\% bovine serum albumin. Then the cells were gently scrapped off with a rubber policeman and adjusted to 2 to 3 x 10\(^6\) cells per mL. One hundred microliters of this cell suspension was mixed with 100 \(\mu\)L of a 0.5\% suspension of IgG-sensitized red cells and centrifuged at 120 x g for ten minutes at 4\(^\circ\)C. After 1 hour of incubation in an ice bath, the pellet was gently resuspended and the cells were examined by phase contrast microscopy. A minimum of 200 cells was counted, and a cell having three or more red cells attached was scored as a rosette. Results are expressed as percent rosette-forming cells (\% RFC) or as attachment index (ie, the average number of red cells attached per Mo). The attachment index (AI) has been estimated by including all monocytes with one, one, or more than one erythrocyte attached and is calculated according to the following formula: AI = total number of red cells attached to 200 Mo divided by 200.

For competitive inhibition of rosette formation, 50 \(\mu\)L of Mo (6 x 10\(^6\) Mo/mL) and 50 \(\mu\)L of IgG-sensitized red cells (1\% suspension) were mixed with 100 \(\mu\)L of F VIII (2 IU/mL) or polymeric IgG (10 mg/mL) and treated as described above.

Determination of CR3. Expression of the complement receptor CR3 on monocytes was assessed by indirect immunofluorescence. Indirect immunofluorescence was carried out by first incubating (30 minutes, 4\(^\circ\)C) 5 x 10\(^6\) monocytes in 70 \(\mu\)L wash medium (Dulbecco's PBS containing 0.1\% Na\(_2\) and 2\% fetal calf serum) with saturating amounts of the monoclonal antibody (MoAb) Mol (94 ed, kindly provided by Dr R.F. Todd), which is directed against the iC3b receptor (CR3) on monocytes, granulocytes, and null cells.\textsuperscript{14} After the first 30-minute incubation period, the cells were washed and again incubated for 30 minutes at 4\(^\circ\)C in wash medium containing an excess of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin (GAM-FITC, Coulter Immunology, Hialeah, FL). After a further wash step, flow cytometric analysis of monocyte cell-surface fluorescence was performed by using a FACS 440 cell sorter equipped with an argon ion laser operated at 488 nm and 270 mW and interfaced to a Consort 30 computer (Becton Dickinson, Mountain View, CA). Contaminating lymphocytes were excluded by preselecting a gate on the forward-angle light scatter v the.

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**Table 1. Characterization of the F VIII Concentrate Studied**

<table>
<thead>
<tr>
<th>Total protein*</th>
<th>Immunoglobulin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1896.0</td>
<td></td>
</tr>
</tbody>
</table>

IgG | 177.3
IgA | 32.4
IgM | 15.2

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>(IU F VIII/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.53</td>
<td></td>
</tr>
</tbody>
</table>

F VIII R:Ag‡ | 51.75
F VIII C‡ | 23.8

Total protein content, immunoglobulin concentrations, and F VIII R:Ag were determined using standard procedures; F VIII C was assessed by a two-stage coagulation assay.\textsuperscript{11}

*mg/1000 IU F VIII.
‡IU/mL.

**Table 2. The Modulatory Activity of F VIII Concentrates Is Confined to a High Mr Fraction**

<table>
<thead>
<tr>
<th>Mo Pretreated with</th>
<th>F VIII Activity</th>
<th>Protein Content (mg/dL)</th>
<th>% Rosette* Forming Cells</th>
<th>Attachment Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>---</td>
<td>77 ± 3.5</td>
<td>3.16 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Dialysis buffer</td>
<td>---</td>
<td>74 ± 3.6</td>
<td>2.73 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>F VIII, 2 IU/mL</td>
<td>+</td>
<td>38 ± 3.3</td>
<td>1.70 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>F VIII fractions, †</td>
<td>MR &gt; 1,270,000</td>
<td>9.7</td>
<td>42 ± 4.1</td>
<td>1.91 ± 0.15</td>
</tr>
<tr>
<td>1,270,000-177,000</td>
<td>---</td>
<td>118.0</td>
<td>76 ± 3.0</td>
<td>2.86 ± 0.26</td>
</tr>
<tr>
<td>177,000-100,000</td>
<td>---</td>
<td>52.9</td>
<td>74 ± 4.1</td>
<td>2.74 ± 0.35</td>
</tr>
<tr>
<td>100,000-28,000</td>
<td>---</td>
<td>85.5</td>
<td>73 ± 3.8</td>
<td>2.70 ± 0.34</td>
</tr>
<tr>
<td>28,000-7,000</td>
<td>---</td>
<td>0.98</td>
<td>76 ± 2.5</td>
<td>2.76 ± 0.15</td>
</tr>
</tbody>
</table>

* = SD of three experiments.
†F VIII concentrate was fractionated by gel chromatography using Biogel A-1.5 M.

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right-angle light scatter histogram; dead cells were excluded by their bright red fluorescence after ethidium bromide staining. A minimum of 10,000 cells was counted and the percentage of fluorescent-positive monocytes stained with the experimental antibody (Mol) minus the percentage of cells stained with a control reagent (mouse ascites containing antibodies directed against nonhuman tissue) was calculated.

Assays of monocyte effector functions (chemiluminescence and killing). Monocyte monolayers were washed twice with 0.15 mol/L NaCl. Then the adherent cells were gently scraped off with a rubber policeman and adjusted to 1 x 10⁶ Mo/mL in Hanks' balanced salt solution (HBSS; for chemiluminescence) or HBSS supplemented with 1% gelatine (for killing assays).

Chemiluminescence of monocytes was determined according to the method of Trush et al. In brief, 1 x 10⁶ monocytes were pipetted into a plastic cuvette (Luma Cuvette, 3M, St Paul) and mixed with luminol (Lumanol-1000, Lumac, Medical Products Division/3M, St Paul) to give a final concentration of 63 μmol/L per cuvette. Then opsonized zymosan particles (Zymosan A, Sigma Chemical, St. Louis; opsonized by incubation in the presence of fresh serum [30 minutes, 37°C, shaking water bath]; 0.237 mg/cuvette) or an outdated intramuscular IgG preparation containing IgG (aggr. 10 mg/mL) for one hour 16 hours prior to determination of Fe receptors. Each bar represents the percentage of Mo forming rosettes with antibody-coated red cells (% RFC) or the average number of red cells attached per Mo (attachment index).

The capacity of monocytes to kill bacteria was assessed by a modification of a procedure originally described by Quie and co-workers. Monocytes (1 x 10⁶ in 100 μL) were pipetted into flat-bottomed microtiter plates (Microtest II, Falcon, Oxnard, CA) and mixed with *Escherichia coli* 089:H10 (monocytes to bacteria ratio ~ 2:1). Fresh AB serum (antibodies and complement)—essential for adequate opsonization—was added to give a total volume of 200 μL per microtiter well and a final serum concentration of 1:25. This suspension was incubated with continuous agitation for 90 minutes at 37°C. Incubation of bacteria in the presence of monocytes plus fresh AB serum absorbed with *E coli* to remove anti-*E coli* antibodies was used as a control. At the end of the incubation period the monocytes were lysed with distilled water, and the number of viable bacteria was determined by a plate-counting method. The number of bacterial colonies per plate was determined by using a colony counter (Biotron, New Brunswick Scientific Inc, Edison, NY).

Results are presented as the number of bacteria per plate ± SEM and as percentage of bacteria killed. This percentage was calculated according to the following procedure: the mean number of bacteria surviving in experimental wells was subtracted from the mean number of bacteria surviving in control wells. The difference was then divided by the mean number of bacteria surviving in control wells and multiplied by 100. Control wells contained bacteria plus monocytes plus fresh serum depleted of anti-*E coli* antibodies; experimental wells contained bacteria plus monocytes plus fresh serum.

**Results.** Statistical evaluation of all results was performed using Student's *t* test or Student's *t* test for paired samples.

**RESULTS**

*F VIII* concentrate preparations have the capacity to down modulate Fc receptor expression on normal monocytes of healthy subjects. The data from Fig 1 show that *F VIII* concentrates had the capacity to decrease the number of Fc receptors expressed on the surface membrane of the monocytes of healthy subjects (down modulation of the Fc receptor). Pretreatment of monocytes with *F VIII* concentrate preparations in concentrations within the therapeutic range led to a significant (*P* < 0.001) reduction in Fc receptor expression as assessed by both the decreased percentage of Mo forming rosettes with IgG-coated red blood cells (% RFC) and the reduction in the average number of IgG-coated red blood cells attached per monocyte (attachment index). The *F VIII*-mediated down modulation of Fc receptor expression was comparable to that obtained after pretreating Mo with polymeric (heat-aggregated) IgG, a substance known to down modulate Fc receptors (controls [pretreatment with buffer]: % RFC 81 ± 1, attachment index 3.92 ± 0.16; pretreatment of Mo with *F VIII* [2 IU/mL]: % RFC 40 ± 1, attachment index 1.85 ± 0.09; pretreatment of Mo with polymeric IgG: % RFC 34 ± 3, attachment index 1.46 ± 0.16; results represent *x* ± SD of ten experiments). Fc receptor down modulation was further supported by the frequency distribution curve shown in Fig 2. The number of IgG-coated red cells attached per *F VIII*
The number of IgG-sensitized erythrocytes bound per monocyte FcR expression was assessed using IgG-coated ox red blood cells. Figure 2 shows the percentage of Fc receptor-positive Mo after a 16-hour incubation period.

The decrease in Fc-receptor-positive Mo was achieved by both competitive inhibition of rosette formation (by adding F VIII to Mo together with IgG-coated red cells) and down modulation of the Fe receptor (by incubating Mo monolayers with F VIII for one hour 16 hours prior to rosette formation; Table 3). Our finding that sequential treatment of Mo with polymeric IgG and F VIII and vice versa did not further decrease the percentage of RFC or the attachment index (Table 4) strongly suggested that F VIII concentrates and polymeric IgG down modulated the same type of Fe receptor.

The reduction in the number of Fc-receptor-positive Mo could not be attributed to trivial cytotoxicity, since neither glycopeptide polymers, only minute amounts of polymeric IgG were found to be still monocyte-associated. This 16-hour incubation period was always part of the Fc receptor-modulation protocol. Finally, a possible effect of buffer components present in lyophlized F VIII preparations was ruled out. Pretreatment of Mo with F VIII buffer (ie, buffer containing the same salt and amino acid concentrations as present in F VIII preparations after reconstitution of the lyophlized product with distilled water: 0.034 mol/L sodium citrate, 0.137 mol/L sodium chloride, 0.266 mol/L glycine) at a dilution corresponding to 2 IU of F VIII/mL did not alter the percentage of Fc-receptor-positive cells (control 84 ± 3.4; F VIII buffer 77 ± 6.6, F VIII [2 IU/mL] 51 ± 3.4; x ± SD of four experiments).

The F VIII concentrates and the respective buffer solutions used in this study were provided by Immuno AG, Vienna, Austria. Similar results with respect to down modulation of Fc receptors could also be obtained by using F VIII concentrates of varying purity (1.3 IU to 12 IU per mg protein) from several other manufacturers. Furthermore, no difference between heat-treated and non-heat-treated F VIII preparations could be observed (M.M. Eibl et al, manuscript submitted).

The Fc receptor-modulating component in F VIII concentrates can be separated from F VIII activity. The above mentioned data (F VIII-induced competitive inhibition of rosette formation and down modulation of Fc receptors

### Table 3. F VIII Concentrate Leads to a Decrease in Fc-Receptor-Positive Mo by Both Competitive Inhibition of Rosette Formation and Down Modulation of the Receptor

<table>
<thead>
<tr>
<th>Competitive Inhibition of Rosette Formation</th>
<th>Down Modulation of the Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>% RFC†</td>
<td>% RFC‡</td>
</tr>
<tr>
<td>Control</td>
<td>81 ± 15</td>
</tr>
<tr>
<td>F VIII (2 IU/mL)</td>
<td>39 ± 2.8</td>
</tr>
<tr>
<td>Poly-IgG (1%)</td>
<td>30 ± 6.6</td>
</tr>
</tbody>
</table>

†F VIII was mixed with monocytes and IgG-sensitized red cells immediately before rosette formation.
‡Mo were incubated in the presence of F VIII for one hour 16 hours prior to rosette formation.
§% rosette forming cells.
Results are expressed as x ± SD of ten individual experiments.
Poly-IgG served as a positive control in these experiments.

### Table 4. F VIII and Polymeric IgG Down Modulate the Same Type of Fc Receptor

<table>
<thead>
<tr>
<th>Mo Pretreated with</th>
<th>First Pretreatment</th>
<th>Second Pretreatment</th>
<th>% Rosette* Forming Cells</th>
<th>Attachment Index†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>Buffer</td>
<td>86 ± 0.5</td>
<td>4.17</td>
<td></td>
</tr>
<tr>
<td>F VIII</td>
<td>Buffer</td>
<td>48 ± 1.1</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>F VIII</td>
<td>F VIII</td>
<td>53 ± 1.1</td>
<td>2.79</td>
<td></td>
</tr>
<tr>
<td>Poly-IgG</td>
<td>Buffer</td>
<td>44 ± 4.8</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
<td>Poly-IgG</td>
<td>Poly-IgG</td>
<td>47 ± 5.3</td>
<td>n.d.§</td>
<td></td>
</tr>
<tr>
<td>Poly-IgG</td>
<td>F VIII</td>
<td>49 ± 3.1</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td>F VIII</td>
<td>Poly-IgG</td>
<td>47 ± 4.5</td>
<td>2.21</td>
<td></td>
</tr>
</tbody>
</table>

% x ± SD of six experiments.
†Average of two experiments.
‡Sequential one-hour pretreatments with the respective substances 16 hours prior to rosette formation.
§Not determined.
Fig 3. The capacity of human monocytes to secrete O2 radicals is reduced by treatment with F VIII concentrate. Chemiluminescence induced by ingestion of zymosan (A) or IgG aggregates (B) was assessed 1–6 hours after pretreatment with PBS (C) or F VIII concentrate (D, 5 IU/mL). Chemiluminescence intensity measured in a bioluminescence counter is plotted as counts per minute (cpm) vs time (minutes).

<table>
<thead>
<tr>
<th>Table 5. The Fc-Receptor-Modulatory Activity of the F VIII High Mr Fraction Can Be Removed by Adsorption to Immobilized Protein A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo Pretreated With</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Buffer (negative control)</td>
</tr>
<tr>
<td>F VIII (positive control)</td>
</tr>
<tr>
<td>High Mr fraction of F VIII</td>
</tr>
<tr>
<td>High Mr fraction not adsorbed to immobilized protein A (effluent)§</td>
</tr>
<tr>
<td>High Mr fraction adsorbed to immobilized protein A (eluate)§</td>
</tr>
</tbody>
</table>

All substances were dialyzed against Dulbecco's PBS prior to use.

*mg/mL.
†± SD of four experiments.
‡Tested after elution with low pH.
§After passage through protein A-Sepharose, the volume of the effluent was reduced to give an F VIII activity of –2 IU/mL. The volume of the eluate was reduced to the same extent.

Comparable to the effects of polymeric IgG) already indicated the presence of a contaminating substance (immune complexes or IgG aggregates) in F VIII concentrate preparations. However, the possibility that the observed effects were due to steric hindrance of Fc receptors by F VIII binding to the monocytes could not be ruled out. Therefore F VIII concentrate preparations were subjected to fractionation. Molecular sieving using a Biogel A-1.5 M column yielded five protein peaks, and as the results from Table 2 show, the Fc receptor modulatory activity was confined exclusively to the high Mr fraction (Mr > 1,270,000 daltons). This fraction also included virtually all of the preparation's F VIII activity. None of the other fractions containing fibrinogen, monomeric IgG, or albumin had any effect on Fc receptor expression. The high Mr fraction of F VIII concentrate was then further fractionated by affinity chromatography on protein A-Sepharose, a substance known to interact with the Fc portions of human IgG1, IgG2, and IgG4.24 The data presented in Table 5 clearly demonstrate that only the fraction that could be adsorbed to immobilized protein A had the capacity to down modulate Mo Fc receptors (see Table 5, line 5). This fraction was also found to be completely devoid of F VIII activity. The fraction not adsorbed to immobilized protein A contained all the F VIII activity and had no Fc receptor-modulatory properties. Rough assessment using the F VIII preparation's total protein and IgG content as well as the protein concentration of the fraction bound to and eluted from immobilized protein A (protein A-Sepharose) indicates that about 1.9% of the total IgG in the F VIII concentrate used in this study was present in an aggregated and/or complexed form. Further results to be published elsewhere, however, demonstrate that the down-modulating activity of a given F VIII preparation is independent of the total IgG it contains (M.M. Eibl et al, manuscript submitted). Control experiments using Sepharose not complexed with protein A also demonstrated that the Fc receptor modulatory activity was not removed by passage through Sepharose alone (data not shown).

Effect of F VIII concentrates and F VIII concentrate fractions on monocyte effector functions. Since interactions with and ligation of Fc receptors are known to provide crucial triggering signals for intracellular microbicidal events,25–27 it was of interest to determine whether the F VIII concentrate preparation's total protein and IgG content as well as the protein concentration of the fraction bound to and eluted from immobilized protein A (protein A-Sepharose) indicates that about 1.9% of the total IgG in the F VIII concentrate used in this study was present in an aggregated and/or complexed form. Further results to be published elsewhere, however, demonstrate that the down-modulating activity of a given F VIII preparation is independent of the total IgG it contains (M.M. Eibl et al, manuscript submitted). Control experiments using Sepharose not complexed with protein A also demonstrated that the Fc receptor modulatory activity was not removed by passage through Sepharose alone (data not shown).
depicted control ascites (left panel). After a one-hour pretreatment with buffer (A) or F VIII (2 lU/mL, B) monocyes were stained with the MoAb Mo1 (right panel) or with control ascites (left panel).

VIII-concentrate-mediated down modulation of Fc receptors would also modify monocyte effector functions. The data depicted in Fig 3 show that a one-hour pretreatment of Mo with 5 IU of F VIII concentrate 16 hours prior to the chemiluminescence assay led to a substantial reduction in the capacity of treated Mo to secrete O2 radicals as compared to controls. The luminol-enhanced chemiluminescence was found to be reduced following stimulation of Mo by both opsonized zymosan and IgG aggregates.

The reduction of IgG aggregate-induced chemiluminescence can easily be explained by a decrease of Fc receptors in the Mo membrane. Chemiluminescence in response to zymosan is also decreased, but this substance stimulates the release of O2 radicals by a pathway independent of Fc receptors, ie, following interaction with receptors for the complement cleavage product iC3b (CR3). However, as can be seen in Fig 4, the expression of this receptor is also substantially diminished following preincubation of human monocyes with F VIII concentrate preparations. In that respect the Fc receptor-modulating component of F VIII concentrates behaves similarly to IgG polymers.

In addition, pretreatment of Mo with F VIII concentrates also significantly down modulated these cells' capacity to kill microbes. E coli 089:H10, an E coli strain that requires opsonization by both antibodies and complement for proper elimination, was used as a model system because this microorganism is easy to handle and is a potentially opportunistic pathogen. The data from Table 6 demonstrate that pretreatment of Mo with varying concentrations of F VIII concentrate preparations led to a dose-dependent reduction of the monocyes' bactericidal capacity (percent killing: controls [Mo pretreated with buffer] 64%, Mo pretreated with 2 IU/mL F VIII 50%, Mo pretreated with 5 IU/mL F VIII 34%, Mo pretreated with 10 IU/mL F VIII 22%). Similar results could be obtained if Mo were pretreated with fraction 1 (high Mr fraction [Mr > 1,270,000] eluting off the Biogel column after molecular sieving of F VIII concentrates). After pretreatment with fraction 1, the killing capacity of Mo was reduced to 36%, as compared to 64% obtained with control Mo (see Table 6, line 6).

**DISCUSSION**

Pretreatment of human monocyes with commercial F VIII concentrate preparations resulted in a down modulation of monocyte functions, as expressed by a reduction of Fc receptors and CR3 in the monocyte membrane, decreased release of oxygen metabolites following stimulation with opsonized zymosan and aggregated IgG, as well as impaired killing of bacteria.

**Table 6. F VIII Pretreatment Reduces the Bactericidal Capacity of Human Monocytes**

<table>
<thead>
<tr>
<th>E coli incubated in the Presence of</th>
<th>Mo Pretreated with*</th>
<th>No. of Bacteria Surviving†</th>
<th>% Killing‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement, monocyes; no anti-E coli antibodies (control wells)</td>
<td>Buffer</td>
<td>731 ± 43</td>
<td>—</td>
</tr>
<tr>
<td>Antibodies, complement, monocyes (experimental wells)</td>
<td>Buffer</td>
<td>265 ± 10</td>
<td>64</td>
</tr>
<tr>
<td>Antibodies, complement, monocyes (experimental wells)</td>
<td>F VIII, 2 IU/mL</td>
<td>362 ± 43§</td>
<td>50</td>
</tr>
<tr>
<td>Antibodies, complement, monocyes (experimental wells)</td>
<td>F VIII, 5 IU/mL</td>
<td>485 ± 40∥</td>
<td>34</td>
</tr>
<tr>
<td>Antibodies, complement, monocyes (experimental wells)</td>
<td>F VIII, 10 IU/mL</td>
<td>571 ± 65∥</td>
<td>22</td>
</tr>
<tr>
<td>Antibodies, complement, monocyes (experimental wells)</td>
<td>Fraction 1 (2.2 IU F VIII/mL)</td>
<td>469 ± 21∥</td>
<td>36</td>
</tr>
</tbody>
</table>

*Mo were pretreated with buffer or F VIII for one hour 16 hours prior to determination of the monocyes' bactericidal capacity.

†Number of bacteria surviving after a 90-minute incubation period, x ± SEM of eight experiments.

‡% killing was calculated according to the following formula:

\[
\text{% killing} = \frac{\bar{x} \text{ number } E \text{ coli in control wells} - \bar{x} \text{ number } E \text{ coli in experimental wells}}{\bar{x} \text{ number } E \text{ coli in control wells}} \times 100
\]

Significant (§P < 0.05 and ∥P < 0.001) increase as compared to the number of bacteria surviving in the presence of monocyes (pretreated with buffer), antibodies, and complement.
The first observation was that F VIII preparations inhibited rosette formation when added to Mo together with IgG-coated red cells. Since IgG-coated red cells are known to interact with the receptor for the Fc portion of IgG, these findings indicated a competition of two similar ligands for the same binding site. Furthermore, polymeric (heat-aggregated) IgG and F VIII concentrates behaved very similarly in competitively inhibiting rosette formation as well as in down modulation of the Fc receptor. This down modulation was achieved by a short (one-hour) treatment of monocyte monolayers with therapeutic concentrations of F VIII 16 hours prior to the respective assays and was comparable whether heat-treated or non-heat-treated F VIII preparations were applied. Sequential treatment of Mo with polymeric IgG and F VIII concentrates did not increase the effect. Fractionation of F VIII concentrate preparations then clearly showed that the modulating activity was confined to a high Mr fraction (Mr > 1,270,000), which showed no F VIII activity and could be purified by affinity chromatography on immobilized protein A.

The molecular nature of this (these) contaminant(s) can be assumed to be (an) immunoglobulin aggregate(s) and/or (an) IgG-containing immune complex(es). In this context it is important to note that, as has already been shown in other studies, monomeric IgG (which can also be detected in commercial F VIII concentrates) is completely inactive in modulating monocyte functions (see Table 2, line 7).

The presence of immune complexes or IgG polymers in F VIII concentrate preparations might have important clinical implications. In a previous study we were able to demonstrate that interaction of monocytes and polymeric (heat-aggregated) IgG led to a down modulation of the monocytes' capacity to release O₂ metabolites (in response to triggering by both aggregated IgG and zymosan) and to kill microbes. In the present paper we report a comparable effect induced by a high Mr component present in commercial F VIII concentrates. Immune complexes or IgG aggregates have also been shown to interfere with immune defense mechanisms in vivo. Recently Virgin and Unanue provided direct evidence for a correlation between the presence of circulating immune complexes and decreased resistance to infection. Using Listeria monocytogenes infection of mice as a model system, these authors demonstrated that in animals previously immunized with an unrelated antigen, secondary antigenic challenge led to a dramatically decreased resistance to the intracellular pathogen. The finding that the decrease in resistance could only be observed if antigenic challenge and infection were given together or not further apart than one day points toward an influence of immune complexes on the early phase of infection. The presence of high levels of circulating immune complexes has also been shown to be one of the major factors for the predisposition to viral and bacterial infections in patients with rheumatoid arthritis. These in vivo data as well as the results from our in vitro studies thus make it conceivable that—together with the already increased level of circulating immune complexes frequently observed in hemophiliacs—the continuous inoculation of immune complexes or IgG aggregates caused by F VIII concentrate treatment contributes to the hemophiliacs' increased susceptibility to opportunistic infections.

Recent clinical evidence indeed supports the possibility of an underlying immune deficiency in hemophiliacs. Children with bleeding disorders who had accidentally been exposed to tuberculosis in a hospital ward showed an unusual susceptibility to that disease. These children, who had been regularly treated with F VIII concentrates, were almost equally as susceptible to tuberculosis as children receiving immunosuppressive chemotherapy (38% of the hemophiliacs developed tuberculosis as compared to 48% of the children on immunosuppressive chemotherapy). Furthermore, a defect with respect to antigen presentation by monocytes has been described in both anti-HTLV III-positive and anti-HTLV III-negative hemophiliacs, an effect that could also be achieved by pretreatment of monocytes from healthy subjects with therapeutic concentrations of F VIII concentrates (manuscript in preparation).

The in vitro data reported in the present publication strongly suggest that treatment with F VIII concentrates might contribute to an impairment of host-defense mechanisms in hemophiliacs. The bleeding disorder that comprises the major clinical problem in hemophiliacs is mainly treated by application of F VIII concentrates. Despite the availability of heat-treated F VIII concentrate preparations, which more or less rule out a de novo infection with HTLV III, the possibility of inducing an immunosuppressed state by this treatment should still be kept in mind. Live HTLV III has been found in the circulation of hemophiliacs, and since the virus is difficult to detect, it might be present in many more of the patients but inhibited from multiplying by a still functioning immune system. This delicate balance could be impaired by F VIII preparations contaminated with immune complexes and/or IgG aggregates. Therefore hemophiliacs now treated with heat-inactivated F VIII concentrate preparations should also be closely surveyed for immunologic functions and the possible outbreak of opportunistic infections. In addition, it appears desirable that F VIII concentrates free of immunosuppressive high mol wt contaminants should become available.

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REFERENCES


A component of factor VIII preparations which can be separated from factor VIII activity down modulates human monocyte functions

MM Eibl, R Ahmad, HM Wolf, Y Lin nau, E Gotz and JW Mannhalter