Comparable Modulation of Human Monocyte Functions by Commercial Factor VIII Concentrates of Varying Purity

by J.W. Mannhalter, R. Ahmad, H. Leibl, J. Göttlicher, H.M. Wolf, and M.M. Eibl

Our previous observation on immune modulation induced by a given factor VIII (F VIII) concentrate preparation was extended by showing that the immune-modulating capacity is a more general feature of F VIII products and is independent of product purity. Interaction of human monocytes with therapeutic concentrations of various F VIII concentrates (0.2 to 2 IU F VIII/mL, six different F VIII concentrates from four manufacturers) led to a significant reduction in the expression of IgG Fc receptors in the membrane of these cells (F VIII concentrate-induced downmodulation of the receptor). This Fc receptor downmodulation was achieved by a short (1-hour) incubation of human monocytes with F VIII concentrates 16 hours prior to the Fc receptor assay and did not correlate with the respective product's IgG content. Although the IgG concentrations of the different products varied greatly (from 1.0 to 177.3 mg/1000 IU F VIII), all products behaved comparably with respect to Fc receptor downmodulation (F VIII-treated monocytes: 34% ± 7% to 44% ± 4% rosette-forming cells; controls in the absence of F VIII: 83% ± 5%). Furthermore, we also were able to demonstrate that heat treatment of F VIII, now used by virtually every manufacturer to eliminate contaminating viruses, had no effect on the respective products' Fc receptor-modulating capacity. The immune-modulating component was characterized as being a high-molecular-range compound containing IgG, IgM, F VIII, and blood group substances (most likely a combination of immune complexes and immunoglobulin aggregates). This compound is present in comparable amounts in both high-purity and intermediate-purity products and apparently copurifies with F VIII during the manufacturing process.

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THE INTRODUCTION of factor VIII (F VIII) concentrates has greatly improved the treatment of patients with hemophilia A, but therapy-mediated side effects have always been of concern. Immediate adverse reactions to infusions of F VIII are known to occur, even though infrequently, whereas long-term side effects such as hypertension, hemolysis, thrombocytopenia, generation of F VIII inhibitors, as well as diseases due to contaminating viruses (eg, non-A, non-B hepatitis, AIDS and AIDS-related complex) play a major role in morbidity and mortality in this group of patients. Contaminating proteins such as transferrin, fibrinogen, immunoglobulins and immune complexes, or immunoglobulin aggregates have been discussed as factors that may contribute to the observed phenomena.

In a recent study, we reported on immune-modulating properties of F VIII concentrates. Interaction of therapeutic concentrations of F VIII with monocytes from healthy subjects led to a downmodulation of receptors for the Fc part of IgG in the membrane of these cells. This downmodulation of Fc receptors was also accompanied by a decrease in monocyte functions, such as the generation of O2 radicals and bacterial killing.

Because of the great practical importance of a possible therapy-mediated immune modulation in the treatment of patients with hemophilia, we investigated six F VIII concentrate preparations with respect to their immune-modulating capacity. The logical question to ask was whether immune modulation correlates with product purity (ie, content of contaminating proteins, especially IgG) or with the heat treatment of F VIII concentrates now used by virtually every manufacturer to increase product safety with respect to transmission of viruses (non-A, non-B hepatitis; HIV). In this article, we report that the downmodulation of monocyte functions observed after interaction with F VIII concentrate (decrease in the expression of monocyte plasma membrane Fc receptors, diminished Fc receptor-mediated phagocytosis) was independent of the product's immunoglobulin content. In addition, our results also demonstrate that heat treatment of F VIII does not result in increased immune-modulating activity. Finally, attempts were made to characterize the immune-modulating components in F VIII concentrates further.

MATERIALS AND METHODS

Factor VIII concentrates. Six commercially available factor VIII (F VIII) concentrates from four manufacturers were used in this study: Faktor VIII HS Behringwerke 500 (lot no. DO 35; Behring), Koate 500 (lot no. NC-8501; Cutter), Faktor VIII HT Hyland, 250 I.E. (lot no. 840628AH11A; Hyland-Travenol), Faktore Antienmofilo (Umano) Hemofil, 960 IU (lot no. 840319AH11A; Hyland-Travenol), Kryobulin (lot no. 09M09083; Immuno AG) and Kryobulin Tim 3 (lot no. 09A068602S; Immuno AG). Three of these preparations were thermoinactivated (Faktor VIII HS Behringwerke 500; Faktor VIII HT Hyland, 250 I.E.; Kryobulin Tim 3).

F VIII preparations were dissolved and adjusted to the appropriate concentrations in RPMI 1640 medium (Flow Laboratories, Irvine, England) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL) and l-glutamine (2 mmol/L) (RPMI supplemented).

Thermoinactivated F VIII concentrates prepared from a pool of blood group-matched donors (Kryobulin Tim 2, lot no. 09M018701S; Kryobulin Tim 3, lot no. 09A128707S) as well as F VIII cryoprecipitates prepared from single donors were also investigated.

Fractionation of F VIII concentrates. F VIII concentrates were first fractionated by affinity chromatography using protein A-Sepharose as described previously. The protein-containing fractions eluting off the protein A-Sepharose column were further fractionated using DEAE-Sephadex A-25 as described previously.

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subjected to molecular sieving [Pharmacia FPLC system; Superox 6 prep grade column HR 16/50, previously calibrated with standard proteins; column-buffer Dulbecco's phosphate-buffered saline (D-PBS) containing 0.1% sodium azide, flow rate 0.5 mL/min]. Protein fractions with a mol wt >400,000 daltons were pooled, extensively dialyzed against D-PBS, and concentrated by ultrafiltration.

Polycrylamide gel electrophoresis (PAGE) and Western blotting. Sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli \(^{28}\) with minor modifications (4% to 12% gradient gel; anodic buffer 0.025 mol/L Tris, 0.2 mol/L glycine, 0.1% SDS; pH 8.3). Samples containing 4 to 7 μg protein were applied, and the gel was run for 6 hours at 20 mA constant current. The separated protein bands were visualized by Coomassie brilliant blue. \(^{27}\)

Following SDS-PAGE, the separated proteins were transferred to nitrocellulose (45-μm, Bio-Rad, Richmond, CA) with the use of a Bio-Rad transblot cell (conditions: 18 V, 0.06 A, 4°C, 16 hours). \(^{24}\)

For immunodetection, rabbit anti-human IgG, rabbit anti-human IgM (Dako, Glostrup, Denmark), and rabbit anti-F VIII-related antigen (Immuno) were used as the first step reagents, followed by an immunoblot assay kit (goat anti-rabbit IgG horseradish peroxidase, Bio-Rad) for the second step.

Hemagglutination assays. Standard hemagglutination assays \(^{29}\) were applied to test for the occurrence of blood group substances and isoagglutinins in the Fc receptor-modulating components of F VIII. Blood groups A-positive and B-positive test erythrocytes, the human anti-A or anti-B reagents, as well as the Coombs sera used in these assays were provided by Immuno AG.

Isolation of mononuclear cells and preparation of monocyte (Mo) monolayers. Mononuclear cells (MCNs) were separated from the peripheral blood of healthy human volunteers by buoyant density-gradient centrifugation. \(^{30}\) Isolated MCNs were suspended in RPMI supplement containing 15% pooled, heat-inactivated (30 minutes, 56°C) AB serum (complete medium), and the monocyte fraction thereof was purified as described. \(^{24,31}\) The purity of the monocytes was >90% as judged by nonspecific esterase staining. \(^{22}\)

Pretreatment of Mo monolayers with F VIII. Immediately after adherence, monocytes were incubated with F VIII concentrates for 1 hour at 37°C in a CO\(_2\) incubator. If not stated otherwise, the F VIII concentration used was 2 IU/mL; concentrations of the various F VIII fractions tested are indicated in the Results section. Treatment of monocytes with heat-aggregated IgG (prepared as described previously)\(^{32}\), a substance known to downmodulate monocyte Fc receptors, \(^{33,34}\) was always included as a positive control. Incubation of monocytes with RPMI supplemented served as a negative control. At the end of the incubation step, the substances used for pretreatment were aspirated, and the monocyte monolayers were washed three times with 0.15 mol/L NaCl. Complete medium was then added, and the cells were incubated for 16 hours at 37°C in a CO\(_2\) incubator. Cytotoxicity tests (performed by trypan blue exclusion) revealed that the abovementioned pretreatment did not decrease the monocytes' viability by >10%.

Determination of Fc receptor and Fc receptor-mediated phagocytosis. IgG-coated ox RBCs were used to determine the expression of receptors for the Fc portion of IgG in the membrane of human monocytes (by rosette formation) \(^{35}\) and to assess the monocytes' phagocytic capacity. Details of both procedures are extensively described in references 24 and 33. For competitive inhibition studies, rosette formation of monocytes with IgG-coated erythrocytes was assessed in the presence of F VIII \(^{24}\).

Results of Fc receptor (FcγR) expression are depicted as percentage of rosette-forming cells (% RFCs) or as attachment index (AI; average number of erythrocytes bound per monocyte). The capacity of human monocytes to form rosettes with IgG-coated RBCs was examined by phase-contrast microscopy. In this process, a minimum of 200 cells was counted, and monocytes with three or more erythrocytes attached were scored as rosettes. In contrast, the AI was estimated by including all monocytes with no, one, or more than one erythrocyte attached and is calculated according to the following formula: AI = total number of RBCs attached to 200 Mo divided by 200. Phase-contrast microscopy was also used to assess the monocytes' phagocytizing capacity (percentage of monocytes having one or more IgG-coated erythrocytes ingested).

Determination of immunoglobulin and protein concentration. IgG, IgA and IgM contents of F VIII concentrates were determined by single radial immunodiffusion (LC-Partigen IgG, IgA, IgM; Behringwerke AG, Marburg, FRG). Protein concentrations were assessed according to the method of Lowry \(^{36}\) or by measuring the OD at 280/260 nm according to the procedure of Warburg and Christian. \(^{37}\)

Statistics. Statistical analysis was performed using Student's t test or Student's t test for paired samples.

**RESULTS**

**Fc receptor-modulating capacity of F VIII concentrates is independent of the preparation's total IgG and protein contents.** The F VIII concentrate preparations studied varied greatly with respect to product purity as well as with respect to the concentrations of contaminating immunoglobulins (Table 1). These variations had no effect, however, on the immune-modulating capacity of the various F VIII preparations tested (Tables 2 and 3). Although the IgG content of the preparations varied from 1 to 177.3 mg IgG/1,000 IU F VIII, the immune-modulating activity of these products was essentially the same. Incubation of monocytes from healthy subjects with F VIII concentrations within the therapeutic range (2 IU F VIII/mL) resulted in a significantly reduced expression of FcγR in the monocyte plasma membrane (P < .001). This was indicated by both a reduced number of monocytes forming rosettes with IgG-coated ox RBCs and a decreased AI (average number of erythrocytes bound per monocyte) (% RFCs from 34 ± 7 to 44 ± 4, AI from 2.03 ± 0.46 to 2.29 ± 0.31; values are mean ± SD). Regardless of the IgG content, the FcγR-modulating capacity of the respective products correlated only with F VIII activity (serial dilution experiments are shown in Fig 1; inset clearly demonstrates a dose-dependent FcγR modulation by an F VIII concentrate in the concentration range from 0.25 to 0.01 IU/mL). This suggests a copurification of the immune-modulating component with F VIII.

Decreased FcγR expression in the monocyte membrane following interaction with F VIII concentrates was either the result of receptor downmodulation (pretreatment of monocyte monolayers with F VIII concentrates for one hour, removal of F VIII concentrates by extensive washing, incubation of the monocyte monolayer in complete medium for 16 hours prior to the assay) (Table 2) or was due to competitive inhibition of receptor–ligand interaction (F VIII concentrates being present during rosette formation) (Table 3). These findings were comparable to the effects of heat-aggregated IgG, \(^{33}\) a substance with well-known FcγR-modulating capacity and, as reported in our previous publication, \(^{24}\) were not caused by trivial effects such as cytotoxicity or effects of F VIII buffer components (data not shown).
**Table 1. Characterization of F VIII Concentrates**

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>F VIII Concentrate</th>
<th>Thermo-</th>
<th>IgG*</th>
<th>IgA*</th>
<th>IgM*</th>
<th>Total Protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behring</td>
<td>Faktor VIII HS Behringwerke 500</td>
<td>+</td>
<td>1.0</td>
<td>0.0</td>
<td>1.5</td>
<td>340.0</td>
</tr>
<tr>
<td>Cutter</td>
<td>Koate 500</td>
<td>−</td>
<td>49.0</td>
<td>13.9</td>
<td>7.0</td>
<td>960.0</td>
</tr>
<tr>
<td>Hyland</td>
<td>Faktor VIII-HT Hyland, 250 IE</td>
<td>+</td>
<td>37.9</td>
<td>3.2</td>
<td>9.9</td>
<td>1,190.0</td>
</tr>
<tr>
<td></td>
<td>Hemofil Hyland, 960 IU</td>
<td>−</td>
<td>43.8</td>
<td>2.3</td>
<td>10.1</td>
<td>1,284.4</td>
</tr>
<tr>
<td>Immuno</td>
<td>Kryobulin TIM 3</td>
<td>+</td>
<td>33.6</td>
<td>1.8</td>
<td>5.3</td>
<td>600.0</td>
</tr>
<tr>
<td></td>
<td>Kryobulin</td>
<td>−</td>
<td>177.3</td>
<td>32.4</td>
<td>15.2</td>
<td>1,896.0</td>
</tr>
</tbody>
</table>

*Milligrams per 1,000 IU F VIII.

As shown in Fig 2, F VIII concentrate treatment of human monocytes led to a decrease in FcγR-mediated phagocytosis as well. This decrease was also independent of the respective product’s IgG content.

**Heat treatment of F VIII concentrates does not increase the Fc receptor-modulating capacity.** Heat treatment of F VIII concentrates, now used by virtually every major manufacturer to eliminate contaminating viruses, has always been a matter of concern. Formation of neoantigens or aggregates by this type of treatment have been suggested. Heat-treated or steam-treated F VIII products have been included in this study as well. As shown in Tables 2 and 3 as well as in Figs 1 and 2, heat treatment had no effect on the FcγR-modulating capacity of F VIII concentrates. Even F VIII preparations produced by the same manufacturer and differing merely in the absence or presence of heat treatment behaved comparably in that respect.

**Further characterization of the Fc receptor-modulating component.** In a previous publication, we demonstrated that the FcγR-modulating component of an F VIII concentrate preparation could be separated from F VIII activity by molecular sieving and affinity chromatography on protein A-Sepharose. For further characterization of this component, two F VIII preparations with highly different IgG contents were selected. Product A (Kryobulin S-Tim 3, lot no. 09A428609 S, Immuno) contained 0.52 mg IgG/mL (23.3 mg IgG/1,000 IU F VIII); 0.036 mg IgG/mL (1.4 mg IgG/1,000 IU F VIII) were found in product B (Haemathe HS 500, lot no. 18301, Behring).

First, the protein components containing an Fc portion of IgG were isolated by passage over protein A-Sepharose. Only a small amount of protein—4.1% (product A) and 1.4% (product B) of the starting material—was bound to the column and could be eluted with low pH. The protein A-Sepharose eluate was subsequently subjected to molecular sieving. This fractionation yielded 3 to 4 peaks, one in the 150-kd mol wt range (corresponding to monomeric IgG) and 2 to 3 peaks with a mol wt >200 kd (data not shown). The fractions with mol wt >400 kd were further studied.

The data in Table 4 show that high-molecular-range fractions from both products had the capacity to downmodulate the expression of FcγR in the membrane of monocytes. In contrast to this, as was demonstrated earlier, the fractions containing monomeric IgG had no effect (data not shown). Table 4 also shows that despite the marked differences in the products’ IgG contents the total amounts of the FcγR-modulating material were comparable (milligram of FcγR-modulating component per 1,000 IU F VIII: product A, 2.8 mg, product B, 0.8 mg).

The FcγR-modulating components of both products were further characterized by electrophoretic procedures (Fig 3). Analysis by SDS-PAGE revealed two major bands corresponding to molecular ranges of 950 kd and 150 kd, respectively. In addition, two faint bands with mol wt of 450 kd and 600 kd were recorded.

Following Western blotting and immunodetection, the two major bands could be identified as IgM and IgG, respectively. The two faint bands reacted with an anti-F VIII antiserum (data not shown). Incubations of the Western blots with radioiodinated IgG and IgM myeloma proteins demonstrated that the IgG and IgM components of the FcγR-modulating factor had no anti-immunoglobulin (rheumatoid factor) activity, whereas purified sheep anti-human IgG and sheep anti-human IgM reagents retained their

Table 2. Downmodulation of Human Monocyte Fc Receptor Expression by Various Commercial F VIII Concentrates is Independent of Products’ IgG Content

<table>
<thead>
<tr>
<th>Monocytes Pretreated With</th>
<th>IgG*</th>
<th>Thermo-inactivated</th>
<th>% RFCs</th>
<th>Al</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faktor VIII HS Behringwerke 500</td>
<td>1.0</td>
<td>+</td>
<td>36 ± 1</td>
<td>2.19 ± 0.25</td>
</tr>
<tr>
<td>Kryobulin TIM 3</td>
<td>33.6</td>
<td>+</td>
<td>44 ± 4</td>
<td>2.03 ± 0.46</td>
</tr>
<tr>
<td>Faktor VIII-HT Hyland, 250 IE</td>
<td>37.9</td>
<td>+</td>
<td>37 ± 2</td>
<td>2.06 ± 0.28</td>
</tr>
<tr>
<td>Hemofil Hyland, 960 IU</td>
<td>43.8</td>
<td>−</td>
<td>35 ± 3</td>
<td>2.09 ± 0.50</td>
</tr>
<tr>
<td>Koate 500</td>
<td>49.0</td>
<td>−</td>
<td>40 ± 2</td>
<td>2.29 ± 0.31</td>
</tr>
<tr>
<td>Kryobulin</td>
<td>177.3</td>
<td>−</td>
<td>34 ± 2</td>
<td>2.18 ± 0.32</td>
</tr>
<tr>
<td>Heat-aggregated IgG</td>
<td>−</td>
<td>−</td>
<td>34 ± 7</td>
<td>1.41 ± 0.33</td>
</tr>
<tr>
<td>RPMI supplemented (controls)</td>
<td>−</td>
<td>−</td>
<td>83 ± 5</td>
<td>5.79 ± 0.32</td>
</tr>
</tbody>
</table>

Rosette formation was examined 16 hours after pretreatment of monocytes with F VIII (2 IU/mL), heat-aggregated IgG (10 mg/mL), or RPMI supplemented. Results were expressed as percentage of monocytes forming rosettes with IgG-coated ox erythrocytes (% RFCs) or as attachment index (Al, average number of erythrocytes bound per monocyte) and are the mean ± SD of four individual experiments.

*Milligrams per 1,000 IU F VIII.
IgG-binding capacity after being subjected to the above treatment (SDS-PAGE and Western blotting) (data not shown). Hemagglutination assays demonstrated the presence of both blood group substances and isoagglutinins in the FcyR-modulating component. Blood group antigens were detected by incubation of a 1:100 dilution of anti-A or anti-B reagents (agglutination titer 1:800) with the FcyR-modulating component (in an amount present in 32 IU F VIII), a treatment that completely abolished the reagents’ agglutinating activity. Agglutination of A-positive or B-positive erythrocytes by treatment with the FcyR-modulating component followed by addition of Coombs serum revealed the existence of anti-blood group antibodies. Such antibodies could also be found in the 150,000 dalton fraction of the Superose 6 prep grade column, thus demonstrating anti-A and anti-B activity in the monomeric IgG contaminants of F VIII as well. This fraction, however, did not contain blood group antigen and was completely devoid of FcyR-modulating activity.

Fc receptor-modulating capacity of immune complexes as well as of F VIII concentrates prepared from blood group-matched donors and F VIII cryoprecipitates prepared from single donors. The following studies were performed to investigate the possibility that immune complexes formed between isoagglutinins and blood group substances may provide a major contribution to the FcyR-modulating activity of F VIII concentrates. The results of these studies first demonstrated that such immune complexes (ICs) are indeed potent modulators of FcyR expression. Incubation of human monocytes with ICs prepared by mixing blood group substances A or AB (Ortho Diagnostics System, Raritan, NJ) with anti-A blood grouping serum (Cooper Biomedical, Malvern, PA) led to a substantial downmodulation of FcyR in the membrane of these cells (monocytes pretreated with A-anti-A IC: % RFCs 35 ± 2, AI 2.3 ± 0.2; monocytes pretreated with AB-anti-A IC: % RFCs 30 ± 5, AI 2.25 ± 0.32; controls: % RFCs 72 ± 2, AI 3.74 ± 0.09; data represent mean ± SD of four to ten individual experiments). Optimal ratios of antigens to antibodies were previously

<table>
<thead>
<tr>
<th>Rosette Formation Performed in the Presence of</th>
<th>IgG*</th>
<th>Thermo-inactivated</th>
<th>% RFCs</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kryobulim TIM 3</td>
<td>33.6</td>
<td>+</td>
<td>42 ± 5</td>
<td>2.15 ± 0.45</td>
</tr>
<tr>
<td>Kryobulim TIM 3</td>
<td>37.9</td>
<td>+</td>
<td>40 ± 4</td>
<td>1.96 ± 0.24</td>
</tr>
<tr>
<td>Hemofil Hyland, 960 IU</td>
<td>43.8</td>
<td>+</td>
<td>37 ± 4</td>
<td>1.75 ± 0.40</td>
</tr>
<tr>
<td>Koate 500</td>
<td>49.0</td>
<td>—</td>
<td>41 ± 3</td>
<td>1.99 ± 0.14</td>
</tr>
<tr>
<td>Kryobulim</td>
<td>177.3</td>
<td>—</td>
<td>40 ± 5</td>
<td>2.01 ± 0.15</td>
</tr>
<tr>
<td>Heat-aggregated IgG</td>
<td>—</td>
<td>—</td>
<td>28 ± 5</td>
<td>1.15 ± 0.17</td>
</tr>
<tr>
<td>RPMI supplemented (controls)</td>
<td>—</td>
<td>—</td>
<td>82 ± 6</td>
<td>5.95 ± 0.09</td>
</tr>
</tbody>
</table>

Percentage of monocytes forming rosettes with IgG-coated ox RBCs (% RFCs) and attachment index (AI, average number of erythrocytes bound per monocyte) were determined in the presence of F VIII concentrates (2 IU/mL), heat-aggregated human IgG (10 mg/mL), or RPMI supplemented (mean ± SD of six experiments).

*Milligrams per 1,000 IU F VIII.

Table 3. Competitive Inhibition of Fc Receptor–Ligand Interaction by F VIII Concentrates

Fig 1. Dose-dependent down modulation of human monocyte Fc receptors by F VIII concentrates. Monocyte monolayers were pretreated for 1 hour with F VIII concentrates followed by a 16-hour incubation period in complete medium. The percentage of rosette-forming cells (% RFCs) was then determined (mean ± SD of four individual experiments). Broken lines represent the normal range (mean ± 2 SD). Thermo-inactivated F VIII concentrates: Kryobulim TIM 3 (○); F VIII-HS Behringwerke 500 (□); F VIII-HT Hyland, 250 IU (△); Kryobulim TIM 3 (×). F VIII preparations without thermo-inactivation: Hemofil Hyland, 960 IU (▲); Koate 500 (●); Kryobulim (■). Total protein and immunoglobulin content of the respective products are shown in Table 1.

Fig 2. Fc receptor-mediated phagocytosis by human monocytes following pretreatment with commercial F VIII concentrates. Phagocytosis of IgG-coated ox erythrocytes by human monocytes was determined 16 hours after these cells were pretreated for 1 hour with F VIII concentrates (2 IU/mL), heat-aggregated IgG (10 mg/mL) or RPMI supplemented (mean ± SD of four experiments). Thermo-inactivated F VIII concentrates (hatched bars); F VIII concentrates without thermo-inactivation (dotted bars).
Table 4. Characterization of the Fc Receptor-Modulating Component in F VIII Concentrates

<table>
<thead>
<tr>
<th>F VIII Characteristic</th>
<th>Product A</th>
<th>Product B</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg IgG/1,000 IU F VIII</td>
<td>23.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Protein content of the Fc receptor-modulating component*</td>
<td>2.8</td>
<td>0.8</td>
</tr>
<tr>
<td>mg/1,000 IU F VIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fc receptor-modulating activity†</td>
<td>25 ± 4†</td>
<td>28 ± 3†</td>
</tr>
</tbody>
</table>

*High-mol-wt fraction (mol wt >400 kd) following affinity chromatography on immobilized protein A and molecular sieving on Superose.
†The % RFCs was determined 16 hours after a 1-hour pretreatment of monocytes with the high-mol-wt fraction described above. Prior to the assay, the samples were adjusted to contain a comparable amount of the immune-modulating component as present in F VIII concentrates at a concentration of 2 IU F VIII/mL.
‡Mean ± SD of six experiments.

determined by checkboard titration (data not shown). Neither antigen alone (% RFCs 71 ± 6, Al 3.64 ± 0.33) nor antibody alone (% RFCs 77 ± 5, AI 3.98 ± 0.26) had any modulatory effect.

If ICs composed of blood substances and isoagglutinins really played a major role in F VIII-induced FcyR modulation, one would assume that F VIII concentrates prepared from blood group-matched donors would be less modulatory. The following results support this hypothesis. Treatment of monocytes with F VIII concentrates prepared from donors matched for blood group A or blood group B (2 IU/mL) resulted in no or only insignificant modulation of FcyR expression (% RFCs (AI) 73 ± 4 (3.7 ± 0.34) and 66 ± 4 (3.25 ± 0.18), respectively; controls 76 ± 4 RFCs, 3.89 ± 0.21 AI; mean ± SD, n = 6).

Results obtained on two single donor cryoprecipitates were inconclusive (cryoprecipitate 1: 74 ± 5% RFCs; cryoprecipitate 2: 32 ± 6% RFCs; mean ± SD, n = 4; controls in the presence of RPMI supplemented 81 ± 7% RFCs). The discrepancies between blood group-matched F VIII concentrates and certain cryoprecipitates could be due to differences in the preparation procedure, but other immune-modulating contaminants potentially present in single donor plasma samples and diluted out in large pools (rheumatoid factors, etc) cannot be excluded.

DISCUSSION

In a previous article, we reported on an immune-modulating component found in a given commercial F VIII concentrate preparation. This compound had the capacity to downmodulate the expression of receptors for the Fc portion of IgG in the membrane of human monocytes, which resulted in an impairment of monocyte functions as expressed by decreased generation of O$_2$ radicals and a reduced ability to kill microbes. Because IgG aggregates exert a comparable effect on monocyte functions, and because we showed previously that the immune-modulating compound from one given F VIII product had the capacity to bind to immobilized protein A, we asked whether there was a possible correlation between the immune-modulating capacity of F VIII concentrates and product purity (especially the content of IgG). Furthermore, possible effects of heat treatment procedures on F VIII concentrate-induced immune modulation were investigated.

To address these points, we tested six F VIII concentrate preparations (of varying IgG content) from four different manufacturers. The results obtained indicated that the observed immune-modulatory capacity was a general feature of F VIII concentrates and did not correlate with the respective product’s IgG content. We were able to show that interaction of monocytes with high-purity products, in which contaminating IgG was barely detectable, led to a decrease in FcyR expression comparable to the decrease observed following interaction of monocytes with F VIII concentrates of intermediate purity. The compound responsible for FcyR modulation was characterized (by affinity chromatography on immobilized protein A, followed by molecular sieving and SDS-PAGE) as being a high-molecular-range compound containing IgG, IgM, and traces of F VIII. This compound shows no rheumatoid factorlike activity (assessed by testing the IgG and IgM binding capacity following SDS-PAGE and Western blotting); however, the presence of blood group substances as well as anti-A and anti-B antibodies was detected by hemagglutination assays. This leads to the hypothesis that ICs between blood group substances and isoagglutinins may play a major role with respect to the immune-modulating capacity of commercially available F VIII concentrates. This hypothesis is supported by our finding that treatment of human monocytes with ICs consisting of blood group substances and the respective blood...
grouping antibodies leads to a marked decrease in FcγR expression. Immune complexes are, as will be shown in another article, very potent modulators of FcγR expression in vitro and exert their effect even at very low concentrations (in the nanogram range) (Mannhalter JW et al, unpublished data). Furthermore F VIII concentrates prepared from blood group-matched donors showed only minimal FcγR-modulating activity. That one cryoprecipitate preparation (from single donor plasma) also modulated FcγR indicates, however, that other factors (eg, immunoglobulin aggregates formed during the manufacturing process) may also contribute to F VIII concentrate-induced FcγR modulation. In conclusion, the immunomodulating compound most likely represents a mixture of ICs and immunoglobulin aggregates present in comparable amounts in both high-purity and intermediate-purity products and apparently copurifies with F VIII during the manufacturing process.

Because transmission of viral infections by F VIII concentrates has become a major problem in the treatment of hemophiliac patients, thermonactivation has been introduced into the production process of F VIII, and heat-treated (or steam-treated) F VIII concentrates are now widely used. However, concern has been expressed that this type of treatment might also increase adverse effects. Especially the possible formation of neoantigens and aggregates has been suggested. The data we presented, however, show that with respect to modulation of mononuclear phagocyte function this concern does not appear to be justified. The three heat-treated F VIII products tested behaved comparably to unbeated F VIII concentrates. Thus, heat treatment does not increase the amount of immune-modulating aggregates in F VIII concentrate preparations.

The possible clinical importance of downmodulated FcγR functions has already been extensively discussed in our previous article and also by other authors. In this context, it is interesting that circulating monocytes of hemophiliac patients also show a decreased expression of FcγR (Mannhalter JW and Eibl MM, unpublished observations), and defects in the antigen-presenting capacity of monocytes isolated from the peripheral blood of these patients have been reported as well. However, despite long-term treatment with F VIII concentrates, hemophiliac patients are generally not especially susceptible to common-type infections. In contrast to this, a decreased resistance to obligatory intracellular pathogens was observed. Immune defense for these types of infections requires properly functioning phagocytes; consequently, an impairment of mononuclear phagocyte functions might render the patients immunocompromised. Thus, to decrease the possibility of therapy-mediated immune modulation in these patients, we suggest that F VIII concentrate preparations free of immune-modulating properties should become available.

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