Production and Characterization of a Murine Monoclonal Antibody Against a Heavy Chain of Hageman Factor (Factor XII)

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A murine hybridoma cell line that produces a monoclonal antibody to human Hageman factor (HF, factor XII) is described. The antibody (P 5-2-1) consists of mouse IgG1 heavy chains and \( \lambda \) light chains, selectively neutralizes HF procoagulant activity, and prevents the proteolytic cleavage of HF during contact activation in plasma. When HF is exposed to P 5-2-1 before the absorption of HF to kaolin, HF procoagulant activity is markedly inhibited. In contrast, P 5-2-1 does not interfere with HF activity after the adsorption of HF to kaolin. P 5-2-1 does not inactivate the prekallikrein-activating activity of 28,000-mol wt HF fragments (HF\(_1\)). P 5-2-1 binds exclusively to the 40,000-mol wt portion of a heavy chain of HF and inhibits the adsorption of HF to negatively charged surfaces. P 5-2-1 immobilized on Sepharose can be used to deplete HF from normal human plasma. This immunoaffinity-depleted plasma is indistinguishable from congenital HF-deficient plasma and can be used as the substrate for HF procoagulant activity assay.

**Materials and Methods**

**Materials**

The following reagents were obtained from sources as indicated: goat antibodies against mouse Fab were made in our laboratory and purified by an affinity column coupled with Fab fragment of mouse immunoglobulin G1; rabbit antibodies against subtypes of mouse heavy and light chains (Miles Lab, Elkhart, Ind); kaolin (Fisher Scientific Co, Fairlawn, NJ); calcium phosphate, chloramine T, and potassium thiocyanate (Wako Pure Chemicals Co, Japan); trypsin (230 U/mg protein; Millipore Corp, Freehold, NJ); lima bean trypsin inhibitor (LBTI, Worthington Biochem Corp, Freehold, NJ); NA\(_{251}\) (Amersham International, England); CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals Inc, Piscataway, NJ); x-ray film (Fuji medical imaging film, type NC, Fuji Photo Film Co, LTD, Japan); polyethylene glycol (PEG, mol wt 4,000, Sigma Chemical Co, St Louis); RPMI 1640 culture medium (Flow Labs, Inc, MacLean, Va); fetal calf serum (FCS, Microbiological Assoc, Bethesda, Md). Other chemicals were reagent grade or better.

**Isolation of HF and HF Fragments (HF\(_1\))**

HF was purified from human plasma as previously described.\(^{14}\) The specific clotting activity was 70 U/mg protein, with 1 unit arbitrarily defined as the amount of activity present in 1 mL of a standard pooled plasma.\(^{14}\) HF gave a single stained protein band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence and absence of a reducing agent. HF\(_1\) (\( \beta-\mathrm{XI}\)a) was prepared from purified HF by digestion with trypsin as previously described.\(^ {15}\) On SDS-PAGE, HF showed a single band (mol wt, 28,000).

**Isolation of Corn Hageman Factor Inhibitor (CHFI)**

CHFI was prepared as reported except that the last step (isoelectric focusing) was omitted. The preparation showed a single component with an apparent mol wt of 14,000 on SDS-PAGE and inhibited the amidolytic activity of HF\(_1\).\(^ {16}\)

**Production of Monoclonal Antibodies**

Murine monoclonal antibodies to human HF were prepared by a modification\(^7\) of the method of Köhler and Milstein.\(^7\) In brief, female BALB/c mice were immunized by intraperitoneal injection...
of 10 μg of purified HF mixed with complete Freund's adjuvant on
day 1, and 10 μg HF in incomplete Freund's adjuvant on day 16. On
day 37, 20 μg HF was given intravenously. Three days later, immune
mouse spleen cells (2.5 x 10⁷) were fused with P3X63Ag8U1 mouse
myeloma cells (2.3 x 10⁷) in the presence of 1 mL 50% PEG-4,000.
Cells were seeded in wells of 24-well culture plates (Nunc, Roskilde,
Denmark) and grown on selective medium (hypoxanthine-ami-
noterin-thymidine [HAT] medium). After 12 days, supernatants
were tested for reactivity against purified HF by a solid-phase
radioimmunoassay using ¹²⁵I-labeled anti-mouse Fab. Briefly, each
well of 96-well plastic flexible plates was coated with purified HF
(10 μg/mL) and blocked by 2% bovine serum albumin (BSA) in
phosphate-buffered saline (PBS). Then, supernatants of hybridomas
were added to the wells. After incubation at 4°C overnight, wells
were washed three times and ¹²⁵I-labeled anti-mouse Fab (20,000 to
40,000 cpm per well) was added. After incubation at room tempera-
ture for four hours, the wells were again washed and the radioactivity
of each plastic cup was counted by a Packard (Downers Grove,
Ill) autogamma-scintillation spectrometer.
The cells in the positive wells were subcloned by limiting dilution
and again screened for the production of antibodies to HF. Sub-
cloned hybridoma cells secreting antibody to HF were injected
intraperitoneally into BALB/c mice for the production of ascites
fluid. Eleven cloned ascites fluid-containing enriched anti-HF were
obtained, and one of them (clone P 5-2-1) was used in the present
study.

Isolation of Immunoglobulin Fraction
of Murine Ascites Fluid

Ascites fluid was adsorbed with calcium phosphate (10 mg/mL)
and kaolin (100 mg/mL), and the supernatant was heated at 56°C
for 30 minutes. The immunoglobulin fraction was then separated
by 45% ammonium sulfate precipitation, reprecipitated again, and
then dialyzed extensively against barbital-saline buffer (BS, 0.025 mol/L
sodium barbital in 0.125 mol/L NaCl, pH 7.4). A murine mono-
clonal antibody to human platelet that had been produced in our
laboratory was used in the present experiments as a control for P
5-2-1. IgG fraction of both P 5-2-1 and control antibody contained
no coagulant activity.

Clotting Assays

The procoagulant activities of HF, factor XI, prekallikrein, high
molecular weight kininogen, factor IX, and factor VIII were measured by
a modified partial thromboplastin time, using congenital deficient
plasmas as substrates. The activities of prothrombin, factor V,
factor VII, and factor X were assayed by a modified one-stage
thrombin generation or stained with Coomassie Brilliant Blue R
(Sigma). Molecular weights were determined by running proteins of known
mol wt (a low mol wt calibration kit, Pharmacia Fine Chemicals) on
gels with the samples.

The Cleavage of ¹²⁵I-HF During the Contact
Activation of Plasma in the Presence
or Absence of P 5-2-1

This was studied by the method of Revak et al. ¹¹⁻¹²⁵I-HF (0.5 ng in
7 μL) was mixed with 10 μL of normal plasma, 11 μL of either P
5-2-1 (10 μg) or control antibody (10 μg) and 172 μL of BS buffer in
a polystyrene tube. The final plasma dilution was 1:20, and the
radioiodinated protein that was added did not increase the plasma
content of HF by more than 1%. Forty microliters of this mixture
was added to 10 × 75-mm glass tubes and shaken at 22°C for five
minutes. The reactions were terminated by the addition of 20 μL 4%
SDS with or without 2-mercaptoethanol, and the samples were then
subjected to SDS-PAGE after boiling for three minutes. The zero-
time sample was obtained by adding the sample directly to the SDS
solution. After SDS-PAGE, the slab gels were fixed, dried, and
subjected to autoradiography using a Fuji medical imaging film
(type NC) with an intensifying screen at ~20°C.

The Effect of P 5-2-1 on the Binding of HF to Kaolin

¹²⁵I-HF (10 μL) was incubated with 10 μL of normal pooled
plasma, 40 μL of either P 5-2-1 (0.02 to 1.10 mg/mL) or control
antibody (1.10 mg/mL) and 40 μL of BS buffer in a polystyrene
tube for 15 minutes at 22°C. Then 40-μL aliquots were mixed with
equal amounts of kaolin suspension (10 mg/mL) for five minutes at
22°C in 1-mL polystyrene Fisher centrifuge tubes. The tube was
then centrifuged for 30 seconds at 5,000 g, and the pellet was
counted for the radioactivity. Binding was expressed as the percent-
age of the total counts that were found in the pellet. Under these
conditions, no significant binding of HF to the polystyrene tubes was
observed.

The Effect of P 5-2-1 on HF Procoagulant Activity
Before and After Kaolin Adsorption

Fifty microliters HF (1.10 μg/mL) was incubated with equal
amounts of either P 5-2-1 (0.88 mg/mL) or control antibody (0.90
mg/mL) for five minutes at 37°C in a 10 × 75-mm polystyrene
tube. Then, 100 μL kaolin (10 mg/mL) was added to the mixture.
After two minutes, 50 μL of either control antibody, P 5-2-1 or BS
was added, respectively, and the mixture was assayed for HF
activity. The results were expressed as the percentage of activity
relative to that found in the control (control antibody and BS).

The Effect of P 5-2-1 on the Prekallikrein-Activating
Activity of HF Fragments (HF₁)

Fifteen microliter HF₁ (31 to 124 ng in 0.025 mol/L BS buffer,
pH 7.4) was incubated with equal amounts of either P 5-2-1 (4
mg/mL BS buffer) or control antibody (4 mg/mL BS buffer) for ten minutes
at 22°C. Twenty-microliter aliquots were then mixed with 50 μL
HF-deficient plasma for two minutes at 0°C. After the action of HF₁,
was blocked by the addition of 5 μL CHF₁ (40 μg/mL BS buffer), a
20-μL portion was incubated with 1 mL of 0.5 mmol/L H-D-
Pro-Phe-Arg-pNA in BS buffer (S-2302, Kabi, Stockholm) for seven
minutes at 37°C. The reaction was terminated by the addition of
acetic acid, and absorbance was read at 405 nm. Preliminary
experiment showed that the activation of prekallikrein in HF-
deficient plasma by HF₁ proceeded linearly under these conditions.
Binding of P 5-2-1 to a Heavy Chain of HF Molecule

Fifty microliters purified HF (21 µg in BS buffer) was cleaved with 4 µL trypsin (0.4 µg) in a total volume of 67 µL at 37 °C for three minutes. After the action of trypsin had been blocked by addition of 40 µL LBTI (0.8 µg in BS buffer), the mixture was divided into two equal portions. One aliquot was incubated with 20 µL Sepharose P 5-2-1 equilibrated with BS buffer and the other with 20 µL Sepharose-control antibody at 0 °C for 30 minutes in a 1-mL polystyrene Fisher centrifuge tube with occasional stirring. The tubes were then centrifuged at 4,000 g for one minute, and the supernatant was separated. The pellets were washed with BS three times and eluted with 4% SDS and 2% 2-mercaptoethanol at 100 °C for three minutes. Both the supernatants and eluates were analyzed by SDS-PAGE. The slab gels were stained with Coomassie Brilliant Blue R.

RESULTS

Characterization of a Monoclonal Anti-HF (P 5-2-1)

P 5-2-1 antibody was found to be IgG2b, 2aa, on immunodiffusion with rabbit antibodies specific for subtypes of mouse heavy and light chains. P 5-2-1 was a homogeneous IgG preparation as analyzed by SDS-PAGE and was derived from a single clone by limiting dilutions. The highest dilution of the IgG fraction (8.8 mg/mL) of P 5-2-1 that gave an unequivocal positive reaction with HF in a solid-phase radioimmunoassay (RIA) was 1.5 x 10⁻³.

When the binding of P 5-2-1 to HF was examined by a solid-phase RIA using [125I]-labeled anti-mouse Fab as described in Materials and Methods, the prior incubation of P 5-2-1 with purified HF or normal plasma inhibited the binding of P 5-2-1 to HF. In contrast, the prior incubation of P 5-2-1 with congenital HF-deficient plasma did not block the binding.

Inhibition of HF Procoagulant Activity by P 5-2-1

When normal pooled plasma was incubated with equal amounts of P 5-2-1 (1 mg/mL) for one hour at 37 °C, the HF clot-promoting activity was reduced to 24% of the control mixture (Table 1). In contrast, the activities of factor XI, prekallikrein, high mol wt-

### Table 1. The Effect of P 5-2-1 on the Activities of Some Clotting Factors

<table>
<thead>
<tr>
<th>Clotting Factors</th>
<th>P 5-2-1 + Normal Plasma</th>
<th>Control Antibody + Normal Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>XII (HF)</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>XI</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>High mol wt kininogen</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>IX</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>VIII</td>
<td>92</td>
<td>100</td>
</tr>
</tbody>
</table>

In a 10 x 75-mm polystyrene tube 50 µL normal pooled plasma was incubated with equal amounts of either P 5-2-1 (1 mg/mL) or control antibody (1 mg/mL) at 37 °C for one hour. The mixture was then diluted ten-fold with BS and was assayed for various clotting factors. The results were expressed as the percentage relative to that of the control mixture.
or not P 5-2-1 influences the activity of HF. In this experiment, HF (0.5 ng in 7 μL) was mixed with 10 μL of normal plasma and 11 μL of either P 5-2-1 (10 μg, lanes 1 to 4) or control antibody (10 μg, lanes 5 to 8) in a total volume of 200 μL. Forty microliters of this mixture was added to glass tubes and shaken at room temperature for five minutes. The reactions were terminated by the addition of SDS with or without 2-mercaptoethanol, and the samples were analyzed by SDS-PAGE. The slab gels were fixed, dried, and subjected to autoradiography. The odd lanes were nonreduced, and the even lanes were reduced samples. The anode is to the bottom. Lanes 1 and 2, P 5-2-1 and no exposure to glass; lanes 3 and 4, P 5-2-1 and five-minute exposure to glass; lanes 5 and 6, control antibody and no exposure to glass; lanes 7 and 8, control antibody and five-minute exposure to glass. *H' and L' indicate the heavy and light chain of HF.

The Effect of P 5-2-1 on the Prekallikrein-Activating Activity of HF Fragments (HFf)

HFf is one form of activated HF (β-XIIa) that retains the active site of XIIa, while losing the surface-binding sites. HFf is a weak coagulant enzyme, but a potent activator of prekallikrein. We tested whether or not P 5-2-1 influences the activity of HFf. In this experiment, HFf activated prekallikrein in HF-deficient plasma to kallikrein, whose activity was then assayed. The experiment was performed. Purified HF was cleaved by trypsin into three fragments, and after trypsin was blocked by LBTI, the tryptic digest was incubated with Sepharose-P 5-2-1 or Sepharose–control antibody. The mixture was then centrifuged, and both the supernatant and eluate from the precipitates were examined by SDS-PAGE (Fig 2). The supernatant from the mixture of native HF and Sepharose–control antibody showed a single band at 80,000 mol wt (Fig 2, lane 3). The supernatant of the mixture of the trypsin-digested HF and Sepharose–control antibody demonstrated an amino-terminal fragment (40,000 mol wt) of a heavy chain and a light chain (28,000 mol wt) (Fig 2, lane 4).

**Table 2. Effect of P 5-2-1 on the Binding of HF to Kaolin**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration (ng/mL)</th>
<th>Bound HF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control antibody</td>
<td>1.10</td>
<td>51.5 ± 1.7</td>
</tr>
<tr>
<td>P 5-2-1</td>
<td>1.10</td>
<td>52.1 ± 1.8</td>
</tr>
<tr>
<td>P 5-2-1</td>
<td>0.28</td>
<td>15.7 ± 2.0</td>
</tr>
<tr>
<td>P 5-2-1</td>
<td>0.07</td>
<td>19.8 ± 1.0</td>
</tr>
<tr>
<td>P 5-2-1</td>
<td>0.02</td>
<td>27.3 ± 0.9</td>
</tr>
</tbody>
</table>

*Mean ± SD of four separate experiments.

Depletion of HF From Normal Plasma by P 5-2-1 Coupled to Sepharose 4B Column

HF could be totally removed from 40 mL of normal pooled plasma by passage over a 3-mL column of Sepharose 4B covalently coupled with P 5-2-1. The drop-through plasma contained less than 1% of HF activity, but essentially normal amounts of all other activities.

**Table 3. The Effect of P 5-2-1 on HF Activity Before and After Kaolin Adsorption**

<table>
<thead>
<tr>
<th>Mixtures</th>
<th>HF Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HF + P 5-2-1 → kaolin → control antibody</td>
<td>12</td>
</tr>
<tr>
<td>2. HF + control antibody → kaolin → P 5-2-1</td>
<td>97</td>
</tr>
<tr>
<td>3. HF + control antibody → kaolin → BS</td>
<td>100</td>
</tr>
</tbody>
</table>

Fifty microliters HF (1.02 mg/mL) was incubated with equal amounts of either (1) P 5-2-1 (0.02 mg/mL) or (2) and (3) control antibody (0.1 mg/mL) and 40 μL of BS for 15 minutes at room temperature. Then 40 μL aliquots were mixed with equal amounts of kaolin suspension (10 mg/mL) for two minutes at room temperature. After centrifugation, the radioactivity in the pellet was counted and binding was expressed as the percent of the total counts that were found in the pellet.

The other fragment (12,000 mol wt) was not observed on the gels, probably due to poor staining of the fragment by the dye. The supernatant from the mixture of the native HF and Sepharose-P 5-2-1 showed no stained band (Fig 2, lane 1), and the supernatant from the mixture of the trypsin-digested HF and Sepharose-P 5-2-1 demonstrated only a light chain (HFf, Fig 2, lane 2). The missing native HF in Fig 2, lane 1, and a 40,000-mol wt fragment in lane 2 were found in the eluate of Sepharose-P 5-2-1 (Fig 2, lanes 5, 6, respectively). The other two stained bands in lanes 5 and 6 were derived from the monoclonal antibody (P 5-2-1) itself (a heavy and light chain of IgG). These data are consistent with the interpretation that P 5-2-1 binds to an amino-terminal 40,000-mol wt fragment of a heavy chain of HF molecule.
clotting factor activities. HF that had been bound to the column was not eluted by 2 mol/L NaCl. It was partially recovered by washing the column with 3 mol/L potassium isothiocyanate. Under these conditions, however, HF seems to be denatured, since it had approximately one third of the procoagulant activity, as compared with the HF antigen measured by a radioimmunoassay.

HF-deficient plasma obtained by an immunoaffinity column was used as an artificial substrate for the measurement of HF activity, and the results were compared with those obtained with congenital HF-deficient plasma (a natural substrate). Plasma from 25 normal individuals and plasma from five individuals with heterozygous HF deficiency were assayed with both substrates (Fig 3). There was good correlation between the assays \( r = .926 \).

DISCUSSION

The present article describes the production and properties of a murine monoclonal antibody (P 5-2-1) against a heavy chain of human HF. This antibody consists of mouse IgG2b heavy chains and \( \lambda \) light chains and selectively inhibits HF procoagulant activity but not the prekallikrein-activating activity of HF.

When normal blood comes into contact with negatively charged surfaces, HF, factor XI, prekallikrein, and high mol wt-kininogen are all adsorbed to the surface and HF becomes enzymatically active. Although the initial event that triggers the contact activation is not yet completely clear, HF undergoes, on the surface, specific limited proteolytic cleavage yielding 52,000-mol wt (a heavy chain) and 28,000-mol wt fragments (a light chain). With prolonged contact activation, a heavy chain appears to be cleaved further into 40,000-mol wt fragments, and a light chain is digested to yield HF1 (\( \beta \)-XIIa). It has been shown that trypsin also cleaves HF into three domains (40,000, 28,000, 12,000 mol wt) and that the 28,000-mol wt portion of the molecule seems to contain an

![Graph](image-url)

**Fig 2.** SDS-PAGE of the supernatant and eluate of the trypsin-digested HF incubated with Sepharose P 5-2-1. Purified HF was cleaved into the heavy (H') and light chain (L') by trypsin as described in Materials and Methods. After the action of trypsin was blocked by LBTI, the mixture was divided into two equal portions. One aliquot was incubated with Sepharose P 5-2-1 and the other with Sepharose-control antibody. The mixtures were then centrifuged, and the supernatant was separated. The pellets were washed and then eluted with SDS and 2-mercaptoethanol. Both the supernatants and eluates were analyzed by SDS-PAGE. The anode is to the bottom. Lane 1, the supernatant of the mixture of native HF and Sepharose P 5-2-1; lane 2, the supernatant of the mixture of the trypsin-digested HF and Sepharose P 5-2-1; lane 3, the supernatant of the mixture of native HF and Sepharose-control antibody; lane 4, the supernatant of the mixture of the trypsin-digested HF and Sepharose-control antibody; lane 5, the eluate of the pellet of the same mixture as lane 1; lane 6, the eluate of the pellet of the same mixture as lane 2.

**Fig 3.** Comparison of HF procoagulant activities measured using either immunoaffinity-depleted plasma or congenital HF-deficient plasma as substrate. Twenty-five normal plasmas and five plasmas from individuals with heterozygous HF-deficiency were assayed with both substrates. The results were expressed as the percentage of HF activity relative to that found in a standard normal pooled plasma (100%).
active site, while the 40,000-mol wt portion is related to surface binding. The mechanisms of inhibition of HF activity by P 5-2-1 were studied. At least two possibilities may be considered as responsible for the action of P 5-2-1 on HF: (1) P 5-2-1 interferes with the binding of HF to surfaces or (2) it blocks the activation and/or activity of the surface-bound HF. When purified HF was mixed with P 5-2-1 before the adsorption of HF to kaolin, HF activity was markedly inhibited. In contrast, P 5-2-1 did not interfere with HF procoagulant activity when HF was exposed to the antibody after the adsorption of HF to kaolin. The failure to inhibit the activity of surface-bound HF by P 5-2-1 could be steric. This possibility, however, was excluded, because P 5-2-1 did not block the activity of HF without surface-binding sites. Furthermore, it was found that P 5-2-1 binds to the 40,000-mol wt portion of a heavy chain of HF and interferes with the adsorption of HF to kaolin. These data are consistent with the first possibility and further extend the observation that 40,000-mol wt fragments are related to the binding of HF to negatively charged surfaces. The precise localization of the binding sites in 40,000-mol wt fragments remains to be studied. In this regard, it is noteworthy that the cell-attachment site of fibronectin was successfully identified by the use of monoclonal antibodies and proteolytic fragments of the molecule. P 5-2-1 would be a useful tool for elucidating the epitope of HF, which is essential for surface binding.

P 5-2-1 was covalently coupled to agarose and was used to selectively deplete HF from normal plasma. The performance of such affinity-depleted plasma in a coagulant assay for HF is as good as that seen with plasma from congenitally deficient patients. No evidence of microheterogeneity of normal HF has been found so far, since all HF was removed from normal pooled plasma (a pool of 24 normal male plasmas). The affinity of P 5-2-1 for HF appears high, since HF was not eluted under mild conditions. Therefore, it was not possible to use the immunoaffinity column for the isolation of HF.

The use of monoclonal antibodies as biospecific and immunochemical probes will be helpful in the study of the structure and function of HF and other coagulation factors.

REFERENCES

Production and characterization of a murine monoclonal antibody against a heavy chain of Hageman factor (factor XII)

H Saito, T Ishihara, H Suzuki and T Watanabe