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Characterization of a monoclonal anti-idiotype antibody to human anti-factor VIII antibodies

(human alloantibodies in hemophilia A/isoelectric focusing of anti-factor VIII antibodies)

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ABSTRACT **Approximately 15% of individuals with hemophilia A develop antibodies (inhibitors) to therapeutically infused factor VIII that interfere with F.VIII coagulant activity. By using isoelectric focusing and immunospecific detection of anti-factor VIII antibodies, inhibitor plasmas showed varied patterns of reactivity characteristic of a polyclonal response. Inhibitor plasma from patient Bt was observed to have an isolated banding pattern, or spectrotype, at pI 8.4 (SP8.4) distinct from his remaining anti-factor VIII antibodies. SP8.4 antibodies from this patient were partially purified and used to prepare monoclonal anti-idiotype antibodies. Monoclonal antibody Mab20-2H was found to detect a spectrotype in isoelectric-focused Bt plasma identical to SP8.4 and to bind anti-factor VIII antibodies. Furthermore, Mab20-2H binding could inhibit the binding of these anti-factor VIII antibodies to antigen, indicating that Mab20-2H recognizes an idiotope associated with antigen binding. Mab20-2H was also found to recognize antibodies from another inhibitor patient. This and other anti-idiotype reagents will be useful for defining genetic factors involved in the human immune response to factor VIII and in designing approaches to prevent or ameliorate this response.**

Hemophilia A is a bleeding disorder, caused by a deficiency or abnormality of the plasma protein factor VIII (F.VIII). This disorder is often treated with transfused F.VIII, purified from pooled normal plasma or produced by recombinant DNA technology (1). Unfortunately, ≈15% of treated patients develop anti-F.VIII antibodies that inhibit F.VIII activity (inhibitors) (2). Since these antibodies interfere with therapy, inhibitor patients create a serious clinical challenge.

The underlying factors that predispose some patients to develop such inhibitors remain unknown. Data on F.VIII gene defects show that a number of inhibitor patients have F.VIII gene deletions, but this is not a dependable predicter of inhibitor development (for reviews, see refs. 3 and 4). A possible association between the immune response genes in the HLA complex and inhibitor formation has also been suggested, but no relationship of predictive value on an individual patient basis has been found (5, 6). Other studies imply that naturally occurring anti-idiotypic antibodies play a role in the regulation of the F.VIII immune response and suggest a clinical applicability of anti-idiotypic reagents for F.VIII inhibitor treatment (7–9).

Anti-idiotype antibodies have also been used as probes to study the specific variable region genes used in an immune response (10, 11). For this reason, investigations of the idiotypic properties of human anti-F.VIII antibodies have the potential of detecting immunoglobulin variable regions associated with inhibitor susceptibility. Polyclonal rabbit anti-idiotype antibodies have been produced that bind to human and murine monoclonal anti-F.VIII antibodies (8, 12). However, such anti-idiotype antibodies have limited use since they detect a number of idiotopes present on the anti-F.VIII antibodies. Precise characterization of single idiotopes on anti-F.VIII antibodies can be studied using monoclonal anti-idiotype antibodies, but such reagents have proven to be difficult to generate. This report describes the preparation of a monoclonal anti-idiotype antibody that reacts with anti-F.VIII antibodies found in two hemophilic patients. Such monoclonal anti-idiotypes against shared idiotypes will be useful for studying the diversity of idiotypic determinants in the human immune response to F.VIII and analyzing the potential role of anti-idiotypes in ameliorating the hemophilic patient's response to F.VIII.

MATERIALS AND METHODS

Isoelectric Focusing (IEF) Analysis of Human Anti-F.VIII Antibodies. The Fab' fragments of anti-F.VIII antibodies from hemophilic inhibitor patient H-01 were immunospecifically purified and radiolabeled with ^{125}I, as described (13).

Plasma samples were subjected to IEF in agarose gels over a pH 3–10 range (Pharmacia) and subsequently diffusion-blotted onto nitrocellulose. The blot was blocked with 5% (wt/vol) bovine serum albumin (BSA), incubated with 0.35 μg of ^{125}I-labeled anti-F.VIII Fab' (1.87 μCi/μg; 1 Ci = 37 GBq) and a source of F.VIII (normal human plasma) or with a negative control (hemophilic noninhibitor plasma containing no F.VIII) overnight at 24°C, and washed. Bound ^{125}I-labeled anti-F.VIII antibody–F.VIII complexes were detected by autoradiography.

Partial Purification of Human Anti-F.VIII Antibodies of pI 8.4 from Plasma of Patient Bt. A saturated ammonium sulfate (SAS) immunoglobulin fraction of Bt plasma was prepared using 50% SAS precipitation and dialysis as described for serum (14). The SAS immunoglobulin fraction was dialyzed into 0.02 M Tris·HCl (pH 8.5) and applied to a DE52 column (Whatman) equilibrated in the same buffer. The protein was eluted in stages with increasing salt concentration of 0.02 M Tris·HCl/0.05 M NaCl and 0.02 M Tris·HCl/0.3 M NaCl, and fractions were pooled. The presence of anti-F.VIII antibodies in the pooled fractions was examined by IEF as above. A fraction enriched with anti-F.VIII antibodies at pI 8.4 was further purified on a Mono P chromatofocusing column (Pharmacia). The protein was eluted using a gradient from pH 9 to pH 6, according to the manufacturer's directions. The purified chromatofocusing fractions were again examined by IEF and detection of anti-F.VIII antibodies. Antibodies from fractions at pI 8.4 (termed SP8.4 antibodies) were pooled and used for immunization.

Anti-Idiotype Hybridoma Production. BALB/c mice were immunized, by intraperitoneal injection, with 25 μg of partially purified SP8.4 antibodies in complete Freund's adju-

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Abbreviations: F.VIII, factor VIII; F.VIII:C, F.VIII coagulant; IEF, isoelectric focusing; BSA, bovine serum albumin; SAS, saturated ammonium sulfate.

vant. A 25-μg booster injection in incomplete Freund's adjuvant was given after 14 days. A final 25-μg injection without adjuvant was given 5 days before the fusion with P3X63-Ag8.653 murine plasmacytoma cells. Fusion and ascites fluid methodologies were similar to that described (15).

Screening for Anti-Idiotype Antibodies. Supernatants from the initial wells were tested for mouse immunoglobulin production by ELISA. Microtiter plates were coated with goat anti-mouse IgA, IgG, and IgM (≈200 μg/75 μl) overnight at 24°C and blocked with 3% BSA. Supernatants from each culture were incubated in the plates for 2 hr at 24°C. The plates were washed four times with 0.02 M Tris·HCl/0.15 M NaCl, pH 8.0 (TBS) with 0.02% Tween 20, and peroxidase-conjugated goat anti-mouse IgG and IgM (7.5 μg/75 μl) in TBS with 1% BSA was added. The plates were developed using a peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and 895 supernatants were found to be positive for immunoglobulin production.

Supernatants from immunoglobulin-producing wells were tested for binding to Bt antibodies. The Bt SAS immunoglobulin fraction (58 μg/12 μl) was isoelectrofocused, diffusion-blotted onto nitrocellulose, and cut into strips. The strips were blocked with 5% BSA, incubated with culture supernatants overnight at 24°C, washed, and incubated with peroxidase-labeled goat anti-mouse IgG and IgM in 1% BSA for 2 hr. The strips were developed with horseradish peroxidase color development reagent (Bio-Rad).

To decrease the total number of isoelectrofocused samples in this initial screening, the following procedure was devised. A positive supernatant in this screening was defined as one that showed any reactivity with the blotted Bt sample. During stage 1, supernatants from 10 or 11 samples were combined and incubated with a blotted sample of Bt SAS fraction. Positive supernatant mixtures were tested in stage 2, with each positive supernatant mixture being divided into two groups of five or six supernatants. The mixtures were diluted 1:2 with 1% BSA and incubated with the blotted sample. The positive supernatant groups were again divided into groups of one or two supernatants and tested in stage 3. All remaining supernatants were tested individually in stage 4. This pyramid type procedure required only 385 blotted samples to identify the positive culture supernatants, in comparison to 895 if all of the supernatants were tested individually.

The 28 positive clones identified in this initial IEF screening were assayed against Bt and normal plasma by the same IEF method to eliminate anti-isotypic and anti-allotypic antibodies. Four putative anti-idiotype reagents were selected by showing a restricted pattern of binding with Bt plasma but no binding to normal plasma. Of the four anti-idiotype antibodies identified, monoclonal antibody Mab20-2H was chosen for further study because it appeared to react with SP8.4 immunoglobulins. Monoclonality was attained by subcloning three times and confirmed by IEF. Mab20-2H was found to be an IgG2a, κ light chain, antibody by ELISA.

HPLC Purification of Mab20-2H. An SAS immunoglobulin fraction was prepared from Mab20-2H ascites. The Mab20-2H SAS fraction was applied to a HPLC DEAE-500 silicon-based column (Perkin–Elmer/Cetus) equilibrated in 0.1 M Hepes (pH 7.5). The sample was eluted with a salt gradient so that the final buffer was 0.1 M Hepes/1.0 M NaCl, pH 7.5.

Affinity Purification of Human Anti-F.VIII Antibodies Using Mab20-2H. HPLC-purified Mab20-2H was coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer's directions. The Mab20-2H resin was incubated with Bt or Lc plasma overnight at 4°C and washed with 0.1 M Hepes (pH 8.0) to remove any unbound protein, and bound antibody was eluted from the resin with 0.0645 M glycine/0.0645 M NaCl, pH 2.5.

One preparation of the affinity-purified Bt antibodies was found to have 9.5 μg of protein per ml by a Bio-Rad protein assay. All preparations of purified Bt antibody appeared to have approximately the same amount of activity by RIA and competitive assays (described below) and were assumed to have the same concentration of protein.

Radiolabeling F.VIII. Purified F.VIII (50 units; supplied by Baxter Laboratories, Duarte, CA) was radiolabeled with Na[^{125}I] by using Enzymobead reagent (Bio-Rad) and purified by gel filtration. The radiolabeled protein had an activity of 0.5 μCi/unit of F.VIII.

RIA to Analyze the Binding of F.VIII to Mab20-2H-Affinity-Purified Bt Antibodies. EIA/RIA strips (Costar) were coated with goat or rabbit anti-human IgG (up to 600 ng/75 μl) for 2 hr at 24°C. The wells were washed once using TBS with 0.02% Tween 20 and blocked with 2% (wt/vol) gelatin. Affinity-purified antibodies diluted in 1% gelatin were added at various concentrations and incubated overnight at 24°C. The wells were washed three times and incubated with ^{125}I-labeled F.VIII (0.95 unit/75 μl) diluted in 1% gelatin overnight. The wells were washed six times and assayed for bound ^{125}I. Percent binding was calculated after the subtraction of background counts (wells that did not contain affinity-purified antibodies).

Competitive Binding Assay to Study the Anti-Idiotype Characteristics of Mab20-2H. The assay was carried out as above except the affinity-purified antibody was added at 75 ng/75 μl. HPLC-purified Mab20-2H was added with the ^{125}I-labeled F.VIII (1.9 units). (The final volume of Mab20-2H and ^{125}I-labeled F.VIII was always 75 μl.) For controls, purified mouse myeloma IgG1 (Sigma) or buffer was used in place of Mab20-2H. The wells were assayed in triplicate. After subtraction of background counts, the results were shown as the percent of inhibition of F.VIII binding by the purified antibody.

IEF Analysis of Mab20-2H Binding to Human Plasma. Plasma or SAS immunoglobulin fractions were focused on an IEF gel with pH 3–10 or pH 8–10.5 gradients (Pharmacia) and diffusion-blotted. The blots were blocked and incubated with Mab20-2H solution overnight. The blots were subsequently washed, incubated with peroxidase- or alkaline phosphatase-labeled goat anti-mouse IgG for 2 hr, washed, and developed.

ELISA Analysis of Mab20-2H Binding to Human Plasma. Microtiter plates were coated with goat anti-mouse IgG + IgM (≈225 ng/ml), washed, and blocked with 20% (vol/vol) normal goat serum. The wells were then incubated with or without Mab20-2H overnight at 24°C, washed, and incubated with 1:100 dilution of human plasma (2 hr). After washing and a 2-hr incubation with peroxidase-labeled goat anti-human IgG (7.5 ng/75 μl), the plates were developed with a peroxidase substrate (Kirkegaard and Perry Laboratories). Binding was calculated after the subtraction of background (wells that did not contain Mab20-2H).

Measurement of F.VIII Inhibitors. Inhibition of F.VIII coagulant (F.VIII:C) activity in Bt plasma and the affinity-purified antibodies was determined using a Bethesda assay with results expressed as Bethesda units/ml (16).

The inhibitor titer of Bt plasma before and after the removal of Mab20-2H-affinity-purified antibodies was determined using a modified Bethesda assay. Equal volumes of pooled human plasma and a dilution of inhibitor plasma were incubated for 1 hr, instead of the customary 2-hr incubation.

Analysis of the Mab20-2H Effect on F.VIII Inhibitors. Purified Mab20-2H (from 1.43 μg/ml to 1.43 mg/ml) and Mab20-2H-affinity-purified Bt antibodies (1 Bethesda unit/ml) were incubated for 1 hr at 37°C. The solutions were stored at −70°C until a standard Bethesda assay was performed.

F.VIII Immunoblot Analysis. Purified F.VIII (6.5 units) was partially degraded by thrombin (0.27 units) at 25°C for 2 hr. The F.VIII was subjected to unreduced SDS/PAGE on

7.5% gels and electrophoretically transferred onto nitrocellulose. The blotted F.VIII was stained for total protein with Aurodye (Janssen Pharmaceutica) by following the manufacturer's directions. The remaining F.VIII blots were blocked for 1 hr with BLOTTO [5% (wt/vol) Carnation nonfat powdered milk/10% normal goat serum/0.1% Tween 20 in 0.014 M sodium phosphate/0.003 M KCl/0.14 M NaCl, pH 7 (PBS)]. The blotted F.VIII was incubated with Mab20-2H-affinity-purified antibodies or a 1:150 dilution of plasma in BLOTTO overnight at 24°C. After three 10-min washes with 0.1% Nonidet P-40 in PBS, the blots were incubated with rabbit anti-human IgG (320 ng/ml) (Jackson ImmunoResearch) in BLOTTO for 2 hr, washed, and incubated with ^{125}I-labeled goat anti-rabbit IgG (0.25 μCi/ml; ICN) for 2 hr. After washing, the blots were autoradiographed.

RESULTS

IEF analysis revealed that inhibitor plasmas have anti-F.VIII antibodies over a wide pI range in a pattern typical of a polyclonal response (Fig. 1). One patient (Bt; lane 9) also had a set of distinct closely spaced bands at pI 8.4, characteristic of the spectrotype pattern seen for a monoclonally derived immunoglobulin and separate from other anti-F.VIII antibodies in this plasma. Because of this distinct spectrotype, called SP8.4, and a high Bethesda unit titer, patient Bt plasma was used as a source of anti-F.VIII antibodies for anti-idiotype production. SP8.4 antibodies were partially purified by HPLC chromatography and chromatofocusing. Since a limited amount of protein (≈75 μg) was recovered, no attempt was made to immunospecifically purify the anti-F.VIII antibodies from other proteins of pI 8.4. The enriched anti-F.VIII antibody preparation was used for immunization in hybridoma production.

To test for the production of anti-idiotypic antibodies, the hybridoma supernatants were examined for binding to isoelectrofocused Bt proteins. Twenty-eight positive clones that demonstrated binding to Bt proteins were identified in this initial IEF screening. The majority of these clones showed binding to both Bt and normal plasma and were assumed to bind to isotypic or allotypic determinants not related to antibody specificity. Four clones, however, showed unique restricted banding patterns when binding to Bt plasma and did not bind to normal plasma, meeting the criteria used to select presumptive anti-idiotype reagents. Since Mab20-2H detected a distinct set of bands at pI 8.4 (Fig. 2, lane 1), it was chosen for further study.

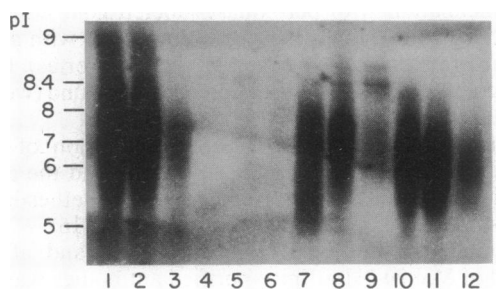

FIG. 1. Detection of human anti-F.VIII antibodies using Western blots of isoelectrofocused plasma. The inhibitor titers (Bethesda units/ml) of the hemophilic inhibitor plasmas were as follows. Lanes: 1, 10,000; 2, 2000; 3, 800; 6, 600; 8, 1000; 9, 1500; 10, not determined; 11, 3000; 12, 500. The titers of the acquired inhibitor plasmas were 4500 (lane 5) and 9000 (lane 7) Bethesda units/ml, and a hemophilic noninhibitor was 0 Bethesda unit/ml (lane 4). Patient Bt plasma is shown in lane 9. Analysis of three bleeding dates for two patients is shown in lanes 1–3 and lanes 10–12. The autoradiograph of this blot was exposed for 72 hr. The control blot, which was incubated without a source of F.VIII, showed no banding patterns.

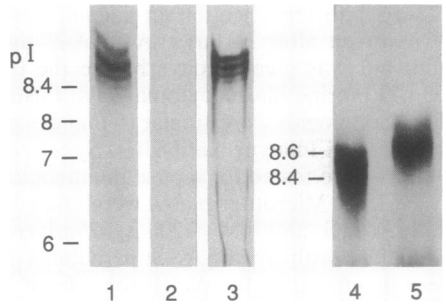

FIG. 2. IEF analysis of Mab20-2H immunoreactivity. Detection of Mab20-2H binding was tested on Western blots of the following plasmas. Lanes: 1, Bt plasma; 2, Bt plasma after treatment with Mab20-2H-immunoaffinity reagent; 3, Bt antibodies bound to Mab20-2H-affinity column and eluted with a low pH buffer; 4, Bt plasma; 5, Lc SAS immunoglobulin fraction. Lanes 1–3 and 4–5 were isoelectrofocused on pH 3–10 and 8–10.5 gradients, respectively.

The set of bands detected by Mab20-2H in isoelectric-focused Bt plasma appeared virtually identical to the SP8.4 pattern (compare Fig. 1, lane 9 with Fig. 2, lane 1). However, the anti-F.VIII antibodies used for immunization were only partially purified, and Mab20-2H could be reacting with any protein of pI 8.4 present in Bt plasma. To determine if Mab20-2H was indeed reacting specifically with a subpopulation of Bt anti-F.VIII antibodies, Mab20-2H was used as a solid-phase immunoaffinity reagent to purify antibodies from Bt plasma. The solid-phase reagent could deplete Bt plasma of antibodies recognized by Mab20-2H (Fig. 2, lane 2). IgG having the characteristic SP8.4 spectrotype could be eluted from the immunoaffinity reagent (Fig. 2, lane 3). The immunoaffinity-purified Bt antibodies bound radiolabeled F.VIII in an RIA, confirming that Mab20-2H binds to SP8.4 anti-F.VIII antibodies in Bt plasma (Fig. 3*A*). The addition of Mab20-2H inhibited the binding of radiolabeled F.VIII to the SP8.4 antibodies by >80% in a competitive binding assay. Control buffer and purified myeloma IgG showed no significant inhibitory activity (Fig. 3*B*). Hence, Mab20-2H is an inhibiting anti-idiotype antibody that binds to a unique idiotope on the SP8.4 antibodies. This idiotope is associated with antigen (F.VIII) binding.

If these idiotope-bearing antibodies represent a clonally restricted population of antibodies, as implied by their IEF spectrotype pattern, then the immunoaffinity-purified SP8.4 antibodies would be expected to detect a unique epitope on the F.VIII molecule. Western blots of thrombin-degraded purified F.VIII were used to compare the epitope specificity of anti-F.VIII antibodies in Bt plasma and the immunopurified SP8.4 antibodies. In Fig. 4, the F.VIII blot stained for protein (lane 1) shows the typical-sized F.VIII fragments of 90, 80, 73, 50, and 43 kDa seen with a partial thrombin cleavage. Lanes 2 and 3 show a pattern of immunoreactivity by whole Bt plasma and affinity-purified SP8.4 antibodies, respectively. Bt plasma has antibodies that bind to the 90, 80, 73, 50, and 43 kDa fragments. SP8.4 antibodies bind to the thrombin-cleaved 43-kDa fragment and its 90-kDa precursor. (With prolonged autoradiography, traces of binding to other F.VIII fragments could be detected, suggesting that the affinity-purified antibodies are not completely pure.) This evidence implies that the SP8.4 antibodies represent a subset of anti-F.VIII antibodies found in Bt plasma and recognize a F.VIII epitope present on the 43-kDa fragment.

The SP8.4 antibodies were also examined for the ability to inhibit F.VIII coagulant activity. The inhibitor titer of Bt:C plasma (1500 Bethesda units/ml) did not change with the removal of SP8.4 antibodies. The affinity-purified SP8.4 antibodies were concentrated 12-fold and were found to have a low inhibitor titer (6 Bethesda units/ml). The addition of

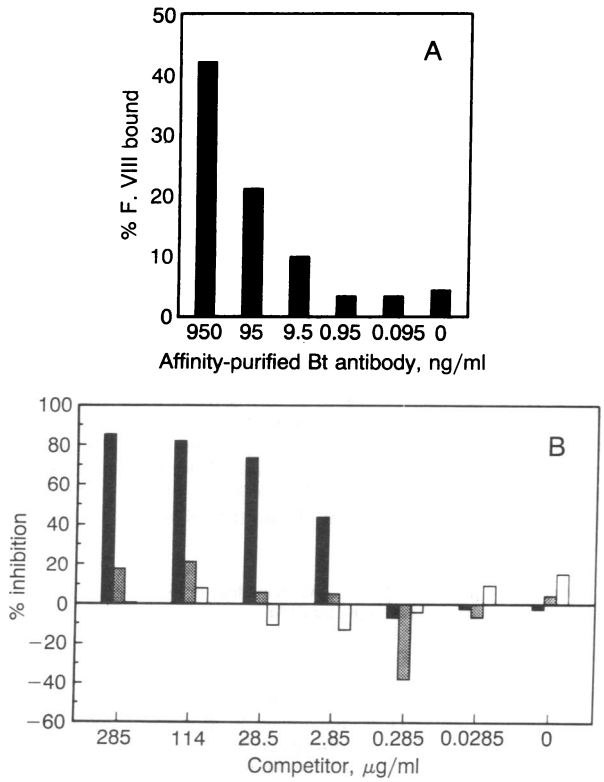

FIG. 3. (*A*) RIA illustrating F.VIII binding to Mab20-2H-affinity-purified Bt antibodies. The exact percent of F.VIII binding can vary slightly with F.VIII and affinity-purified antibody preparations. (*B*) Competitive RIA showing the inhibition of F.VIII binding to the affinity-purified antibodies by Mab20-2H. Solid bars, anti-idiotype antibodies; stippled bars, control antibody; open bars, buffer.

various concentrations of Mab20-2H did not remove this inhibiting activity. This inhibiting activity is likely to result from the above mentioned traces of additional antibody populations in the affinity-purified antibody preparation. Hence, the binding of Mab20-2H-affinity-purified Bt antibodies to F.VIII does not appear to inhibit F.VIII:C activity.

To see if Mab20-2H also binds to antibodies in other plasmas, idiotope-specific ELISAs and IEF assays were developed (Table 1). Noninhibitor or normal plasmas tested showed no significant binding. Of the 52 inhibitor plasmas analyzed, Mab20-2H bound to antibodies present in two inhibitor plasmas, including Bt plasma. In inhibitor plasma from patient Lc, the spectrotype detected by Mab20-2H is at

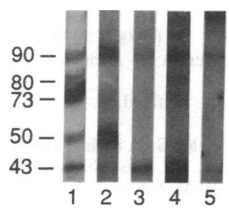

FIG. 4. Western blots of thrombin-cleaved F.VIII. Lane 1 shows protein staining of thrombin-cleaved F.VIII fragments, with molecular masses shown in kDa. The fragment sizes are identified in text and figure as defined (17). On this SDS gel, the molecular masses of the fragments were 91, 77, 69, 51, and 42 kDa. Lanes 2–5 show immunoblots of the binding of anti-F.VIII antibodies to specific thrombin-cleaved F.VIII fragments. Lanes: 2, Bt plasma [2.5-hr exposure; Bt plasma also binds the 80-kDa fragment when an increased amount of this fragment is present (unpublished data)]; 3, Mab20-2H-affinity-purified Bt antibodies (4-hr exposure); 4, Lc plasma (4.5-hr exposure); 5, Mab20-2H-affinity-purified Lc antibodies (96-hr exposure).

Table 1. Analysis of Mab20-2H binding to human plasma

Patient type	Total tested	Tested by ELISA	Tested by IEF	Total positive
Hemophilic inhibitor	49	49*	22	2†
Acquired inhibitor	3	3	2	0
Hemophilic noninhibitor	54	54	6	0
Normal	100	20	94	0

Some plasmas were tested by both ELISA and IEF.
*Two patients developed immune tolerance to F.VIII, and multiple bleeding dates before and after tolerance were negative by ELISA. One patient, tested for immunoreactivity in five bleeding dates from May 1980 to September 1982, has been described (18).
†Multiple bleeding dates for Bt (14-year span) and Lc (5-year span) were positive by ELISA, IEF, or both.

pI 8.6 (Fig. 2, lane 5), slightly higher than the SP8.4 antibodies detected in Bt plasma. Lc antibodies purified using Mab20-2H-immunoaffinity resin were found to bind the 43-kDa fragment of F.VIII and its 90-kDa precursor (Fig. 4, lane 5). (As was the case with affinity-purified Bt antibodies, some binding to other F.VIII fragments could be detected with extended autoradiography and was assumed to be due to contaminating antibodies.) This clonal population of anti-F.VIII antibodies was found to be a subset of the anti-F.VIII antibodies in Lc plasma that bind to the 90-, 80-, 73-, and 43-kDa F.VIII thrombin-cleaved fragments (Fig. 4, lane 4).

DISCUSSION

IEF has proven to be a powerful method for studying the clonal origin of antibodies. A monoclonal antibody derived from a single B-cell clone has a unique IEF pattern, called a spectrotype, which often consists of three to six evenly spaced bands. This pI microheterogeneity of the antibodies is due to post-translational modifications, such as various degrees of glycosylation and deamidation (19, 20). The human immune response to some antigens, including thyroglobulin in the autoimmune disease Hashimoto-type thyroiditis and *Haemophilus influenzae* b capsular polysaccharide, has been shown to be oligoclonal in nature and to have distinct IEF spectrotypes (21, 22). One would expect that each individual banding pattern is derived from a single antibody-producing clone in such a response. Although the human immune response to F.VIII is complex, some patients have unique spectrotypes that appear to be derived from a single antibody-producing clone (Fig. 1). The SP8.4 antibodies in Bt plasma (Fig. 1, lane 9) are presumably monoclonal in origin and were chosen as a target for the production of monoclonal anti-idiotype reagents.

Such monoclonal anti-idiotypes proved difficult to generate even using a partially purified clonally restricted antibody population for immunization. Fewer than 0.5% of the original immunoglobulin-producing clones were candidates for anti-idiotype antibodies. Only four putative anti-idiotype antibodies were produced that detected unique spectrotypes when incubated with isoelectric-focused Bt plasma and not with normal plasma.

Mab20-2H was found to bind to anti-F.VIII antibody populations of pI 8.4 and 8.6 in Bt and Lc inhibitor plasmas, respectively. The antibodies recognized by Mab20-2H in both of these inhibitor plasmas bind the 43-kDa thrombin-cleaved fragment of F.VIII. Since Mab20-2H detects an idiotope associated with antigen binding on the anti-F.VIII antibodies in Bt plasma, it is likely that antibodies from Bt and Lc plasma recognize the same or similar F.VIII epitope on the 43-kDa chain.

To see if the anti-F.VIII antibodies that recognize this F.VIII epitope can neutralize F.VIII:C activity, Mab20-2H was used to affinity-purify antibodies from Bt and Lc plasma.

Some F.VIII:C inhibitory activity could be detected in the preparation of affinity-purified Bt antibodies (0.053 Bethesda units/μg of protein). Although it is possible that this activity may be due to inhibitory activity inherent to the SP8.4 antibodies, it is likely that this represents contamination by polyclonal antibodies in the preparation for the following three reasons. (*i*) With longer exposure of the autoradiograph in Fig. 4, small amounts of anti-F.VIII antibodies to other fragments could be detected, including the 50-kDa chain where Bt antibodies capable of neutralizing F.VIII activity are known to bind (23). (*ii*) By making the assumptions that all inhibitors in Bt plasma are IgG and that most of the IgG in Bt plasma does not have anti-F.VIII:C activity, immunospecific purification of a clonal population of inhibiting antibodies would be expected to yield a higher specific inhibitory activity than found in plasma (13, 24). The specific activity of the Mab20-2H-affinity-purified antibodies (0.053 Bethesda units/μg) was 3.2 times lower than Bt plasma (0.17 Bethesda units/μg). Finally, Mab20-2H, although capable of inhibiting the binding of purified SP8.4 antibodies to ^{125}I-labeled F.VIII, did not inhibit the F.VIII:C neutralizing activity of the preparation. For these reasons it is likely that trace contaminating antibodies account for the Bethesda titer seen in the immunoaffinity-purified preparation of SP8.4 antibodies. Similarly, no F.VIII:C inhibitory activity was detected in the antibodies affinity purified from Lc plasma. Hence, antibodies in Bt and Lc plasma that are recognized by Mab20-2H appear to bind to a nonneutralizing epitope on the 43-kDa thrombin fragment of F.VIII.

Reactivity with the 43-kDa heavy chain-derived fragment is common in hemophilic inhibitor plasmas with at least 50% tested having such activity (ref. 25 and unpublished data). Some of the 43-kDa epitopes are known to be neutralizing, but this is a clear demonstration of a nonneutralizing epitope on this chain (26). Nonneutralizing epitopes have also been reported on the B domain and 80-kDa chain of F.VIII (27, 28). The clinical significance of such nonneutralizing antibodies is unknown although such antibodies have been demonstrated to be present in patients after the induction of tolerance to F.VIII. It has been speculated that these antibodies may somehow play a role in maintaining such tolerance (28). Two inhibitor patients included in Table 1, where tolerance was induced or occurred spontaneously, showed no immunoreactivity with Mab20-2H before or after tolerance (18).

We have demonstrated the feasibility of producing a monoclonal anti-idiotype reagent to shared epitopes present on anti-F.VIII antibodies in hemophilic inhibitor plasmas. Mab20-2H detected antibodies in two of the 52 (3.8%) hemophilic inhibitor plasmas tested. We have also generated three other putative anti-idiotype reagents that detect other antibody populations in Bt plasma. Preliminary work shows that one of these monoclonal antibodies reacts with three inhibitor plasmas and did not react with other inhibitor or normal plasmas. This evidence suggests that this monoclonal antibody also detects a shared idiotope. Hoyer *et al.* (24) have briefly noted the production of monoclonal anti-idiotype reagents that appear to detect public idiotypes. Such public anti-idiotype antibodies would be useful for identifying genetic factors important in the immune response to F.VIII and for potential clinical use.

In clinical trials, anti-idiotype reagents have been useful in the treatment of human B-cell lymphomas (29). Further study with 199 anti-idiotype antibodies showed that similar idiotypes are present on the lymphoma tumors from various patients. Up to 6.2% of the tumors demonstrated shared idiotypy, depending on the antibody evaluated (30). This panel of anti-idiotype antibodies should be useful in the therapy of these patients. The amount of shared idiotypy demonstrated by Mab20-2H is 3.8% in the inhibitor plasmas tested here. The generation of a large panel of anti-idiotype reagents with this or a greater amount of shared idiotypy may also prove to be useful in the therapy of F.VIII inhibitors. Furthermore, the long-term idiotypic stability in the F.VIII immune response, as demonstrated by Mab20-2H (Table 1), makes the development of an anti-idiotype therapy more feasible.

A number of different therapies have been reported for inhibitors including immunoadsorption and tolerizing therapies, which show promise, but there is still no completely satisfactory treatment available (31, 32). A knowledge of the idiotypic properties of anti-F.VIII antibodies may be helpful in the optimization of the above procedures and development of therapies for the hemophilic inhibitor patient.

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