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# IDENTIFICATION OF PLASMA ANTIBODY EPITOPES AND GENE ABNORMALITIES IN JAPANESE HEMOPHILIA A PATIENTS WITH FACTOR VIII INHIBITOR

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# ABSTRACT

Eleven Japanese hemophilia A patients with anti-factor VIII (FVIII)<sup>a)</sup> inhibitors were studied to localize both their inhibitory antibody epitopes and their genotypes. The inhibitor epitopes were studied in nine severe hemophilia A patients by means of a scanning method using the oligopeptide panel covering the FVIII polypeptides without the B domain. The 107 15 mer-peptides were synthesized on solid-phase pins and analyzed for their reactivity with diluted patient plasma. As indicated previously, a series of peptides corresponding to the A2 and C2 domains were recognized by plasma antibodies from 2 patients and 4 patients, respectively. In contrast, all the antibodies bound to several epitopes in the A3 domain, while an epitope 1809-1821 covering the putative factor IX binding site was found in 3 patients. Southern blotting analysis showed that 8 out of 11 patients had either gene deletions or inversions of the FVIII gene, indicating a higher proportion of gross gene alterations in inhibitor-positive hemophilia A patients. However, the correlation of gene abnormality type with epitope location was not fully established.

Key Words: Factor VIII, Hemophilia A, Factor VIII inhibitor, Southern blotting, Gene inversion

## INTRODUCTION

Coagulation factor VIII (FVIII) is the essential cofactor of enzymes involved in the intrinsic activation of the blood coagulation cascade. Circulatory FVIII is synthesized mainly in the liver and is stabilized in combination with the von Willebrand factor (VWF). The 200-92 KDa heavy chain consists of two A domains (A1 and A2) and a large B domain. The B domain does not appear to contribute to the FVIII procoagulant activities.<sup>1)</sup> The 80 KDa light chain has the A3 domain and two C domains (C1 and C2). In the presence of a phospholipid and calcium ion, the activated form of factor VIII (FVIIIa) promotes proteolytic activation of factor X

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<sup>&</sup>lt;sup>a)</sup> FVIII, factor VII

(FX) by activated factor IX (FIXa)<sup>b)</sup>. The binding of FIXa to FVIIIa is a necessary step for FX activation.

Patients with a deficiency of FVIII are diagnosed as hemophilia A, and are usually treated with repeated infusions of FVIII concentrate. The development of anti-FVIII antibodies with neutralizing activity, FVIII inhibitors, presents serious therapeutic complications. While anti-FVIII antibodies are usually found in 6-12% of all hemophilia A patients,<sup>2)</sup> it has been estimated that such antibodies occur in 20-30% of patients with severe forms of the disease.<sup>3)</sup>

There is a great variety of gene abnormalities within the FVIII or FIX gene residing in the X-chromosome of patients with hemophilia.<sup>4,5)</sup> In hemophilia B, a high proportion of patients with anti-FIX inhibitors possesses either gross deletions<sup>6)</sup> or point mutations<sup>7)</sup> resulting in truncated protein products. In hemophilia A, several studies had indicated that the risk of inhibitor development is also related to DNA abnormalities which result in no protein translation, including deletions, inversions and stop mutations.<sup>8-10)</sup>

Domain localization of the inhibitor epitope has been characterized by various techniques. To date, the A2 domain (residues 380-711) and C2 domain (2173-2332) of mature human FVIII are acknowledged to contain major epitopes for anti-FVIII inhibitors.<sup>11-14)</sup> However, the contribution of other factor VIII domains has not been established. To localize detailed epitopes of the plasma antibodies from inhibitor-positive hemophilia A patients, we examined 9 patients using a peptide panel comprised of 107 overlapping 15-mer peptides. Each peptide was synthesized on a solid-phase pin and covered the entire amino acid residues of the B domain-less human FVIII. Furthermore, we studied gene abnormalities using Southern blotting analysis to identify the structural gene alterations in these patients.

## MATERIALS AND METHODS

## Patients

Venous blood was drawn from hemophilia A patients and from 30 normal male volunteers after informed consent had been obtained. We studied 11 severe hemophilia A patients, each with <1% of normal FVIII procoagulant activity. Anti-FVIII antibodies had developed in all the patients after replacement therapy. The titer of inhibitors in Bethesda Units and patient clinical characteristics are summarized in Table 1. We defined any FVIII inhibitor titer of >10 Bethesda Units/ml as a "high responder," while a FVIII inhibitor titer with less than 10 Bethesda Units/ml was a "low responder" (Table 1). Normal-pooled plasma was arbitrarily defined to contain 100% of FVIII.

## FVIII and inhibitor assay

FVIII procoagulant activity was measured by an activated partial thromboplastin time assay with FVIII deficient plasma (George King BioMedical, Overland Park, KA, USA) and Coa SCREENER hemostasia analyzer (Labor, Ahrenburg, Germany). FVIII inhibitor activity was measured in the Bethesda assay. One Bethesda Unit (BU) is equal to 50% residual activity.

## Peptide synthesis for FVIII epitope mapping

An epitope mapping study of anti-FVIII inhibitors was performed as described in our previous study for determining epitopes of anti-FIX inhibitors in hemophilia B patients.<sup>15)</sup> Briefly, we synthesized 107 peptides of 15 amino acids by moving 13 amino acids throughout mature FVIII polypeptides. However, the targeted region which lacked the B domain (712-1648) is less

<sup>&</sup>lt;sup>b)</sup> FIX, factor IX

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functional than the other domains.<sup>1)</sup> The peptide synthesis protocols were based on the principles of solid-phase peptide synthesis using fluorenylme-thoxycarbonyl (Fmoc)-amino acid active esters. Synthesis was performed on prederivatized polyethylene pins as described in the protocols of the Pin technology Epitope Scanning Kit (Cambridge Research Biochemicals, Cheshire, UK). After acetylation of terminal amino groups and side-chain deprotection, peptides were coupled to polyethylene pins and directly assayed for antibody binding by an enzymelinked immunosorbent assay (ELISA)<sup>c)</sup> as described below. The amino acid sequence was deduced from cDNA coding for human FVIII, and the numbering of amino acids was according to Vehar, *et al.*<sup>16)</sup>

## ELISA for inhibitor epitope localization

The pins bearing synthesized peptides were incubated at 25°C with agitation for 1 hour in microtiter plates containing 10 g/L of ovalbumin, 10 g/L of bovine serum albumin (BSA)<sup>d)</sup> and 0.5 g/L of sodium azide in phosphate buffered saline, pH 7.2 (PBS).<sup>c)</sup> The pins were then incubated at 4°C overnight with the patients' plasma diluted 250-1000 times with 10 g/L of ovalbumin, 10 g/L of BSA and 0.5 g/L of sodium azide in PBS. Normal-pooled plasma was diluted 200 times. Pins were washed four times in PBS/0.5% Tween 20 at room temperature with agitation followed by 1 hour incubation at 25°C with anti-human IgG antiserum conjugated with horseradish peroxidase (Amersham, Arlington Heights, IL, USA) diluted 1500 fold. The pins were then washed 4 times in PBS/0.5% Tween 20 at room temperature and incubated in microtiter plates with 0.5 g/L of azino-di-3-ethylbenzthiazolin-sulphonate in 0.2M citrate buffer pH 4.0 containing 0.3 ml/l of hydrogen peroxide at 25°C in the dark with agitation. Once the plates looked as though they had produced sufficient color, the incubation was stopped by removing the pins. Plates were read immediately in a spectrophotometric microplate reader at a wavelength of 405 nm. The assay was performed for each plasma specimen at once to compare the OD value generated. For each plasma specimen, mean OD value testing all the pins was calculated. Peptide pins that showed a value more than mean + 2SD were considered positively bounded by the inhibitor plasma.

# Immunoglobulin subclasses of inhibitory antibodies

The immunoglobulin isotype distribution was measured by a FVIII inhibitor neutralization assay. Sheep antisera to human IgG1, IgG2, IgG3, and IgG4 were obtained from Miles Laboratories, Kankakee, IL. Rabbit antiserum for human  $\kappa$  light chain and  $\lambda$  light chain were from Dako (Glostrup, Denmark). Diluted patients' plasma (adjusted to 1-2 BU/ml of inhibitor titer) was incubated with each sheep antiserum for 2 hours at 37°C to neutralize the inhibitory antibodies. The mixture was then incubated with Normal-pooled plasma, and residual FVIII procoagulant activity was measured as described above. The Bethesda Unit was determined for each antiserum, and the contribution of the IgG subclasses was calculated by normalizing them to the plasma prior to incubation with antiserum.

## Southern blotting

DNA samples were prepared from peripheral blood lymphocytes by established methods. Southern blotting was performed as described.<sup>17)</sup> The following cloned FVIII cDNA fragments were used as probes:<sup>18)</sup> 1) probe A: pKpn-Sac, a 1.8 kb cDNA fragment that spans exons 1-12;

c) ELISA, enzyme linked immunosorbent assay

<sup>&</sup>lt;sup>d)</sup> BSA, bovine serum alubmin

e) PBS, phosphate buffered saline, pH 7.2

| (1:C Inhi<br>(6) (1)<br>(1) 5.5 |              |           | ,    |         | 101      | ,    |          |         |                          |
|---------------------------------|--------------|-----------|------|---------|----------|------|----------|---------|--------------------------|
| 0 3                             | ibitor titer | Inhibitor | -    | gG subc | class (% | _    | Light cr | (%) unu | Structural gene          |
| ŝ                               | BU/ml)       | response  | IgG1 | lgG2    | lgG3     | IgG4 | к        | ٢       | abnormality              |
|                                 | 6            | Low       | 16.2 | 0       | 27.6     | 56.2 | 100      | 0       | Inversion, Distal type   |
| 0                               | 10           | Low       | *    | *       | *        | *    | *        | *       | Inversion, Distal type   |
| 10                              | 00-4000      | High      | 0    | 0       | 0        | 001  | 54.5     | 45.5    | Exon 1-22 deletion       |
| 20                              | )-1200       | High      | 22.2 | 0       | 18.8     | 59   | 100      | 0       | Unknown                  |
| 9                               | 70           | High      | 19.4 | 5.9     | 12.2     | 62.5 | 68.8     | 31.2    | Exon 2-6 deletion        |
| 68                              | 3-500        | High      | 14.1 | 0       | 0        | 85.9 | 69.3     | 30.7    | Inversion, Proximal type |
| 18                              | 30-4000      | High      | 31.8 | 0       | 21.9     | 46.3 | 71.6     | 28.4    | Exon 1-22 deletion       |
| 40                              | )-4000       | High      | 22.6 | 0       | 4,4      | 73   | 100      | 0       | Exon 23-25 deletion      |
| -0                              | Ś            | Low       | *    | ×       | *        | ¥    | *        | *       | Unknown                  |
| .6                              | 25           | Low       | 14.1 | 0       | 9.7      | 76.2 | 76       | 24      | (Not tested)             |
| 24                              | +            | High      | 15.5 | 9       | 9.1      | 69.4 | 21.5     | 78.5    | Exon 2-22 deletion       |

1. 3 . Comments of annihility 15 mor analiston of D domain loss (A1A2A1D3) factor VIII

| ю<br>Хо      | start               | end | No. | start                  | end No       | b. start           | end           | No.      | . start                   | end N  | o. start                              | end  |
|--------------|---------------------|-----|-----|------------------------|--------------|--------------------|---------------|----------|---------------------------|--------|---------------------------------------|------|
|              | 1 ATRRYYLGAVELSWD   | 15  | 23  | 287 EISPITFLTAQTLLM    | 301 4        | 5 573 VILFSVFDEN   | RSWYL 587     | 20       | Z 1796 QRQGAEPRKNFVKPN    | 1810   | 89 2082 HGIKTQGARQKFSSL               | 2096 |
| 2            | 14 WDYMQSDLGELPVDA  | 28  | 24  | 300 LMDLGQFLLFCHISS    | 314          | 6 586 YLTENIQRFI   | PNPAG 600     | 00       | 8 1809 PNETKTYFWKVQHHM    | 1823   | 90 2095 SLYISQFIIMYSLDG               | 2109 |
| ന            | 27 DARFPERVEKSFEN   | 41  | 25  | 313 SSHQHDGMEAYVKVD    | 327 4        | 17 599 AGVQLEDPEF  | .0ASNI 613    | 00       | 9 1822 HMAPTKDEFDCKAWA    | 1836   | 91 2108 DGKKWQTYRGNSTGT               | 2122 |
| 4            | 40 FNTSVVYKKTLFVEF  | 54  | 26  | 326 VDSC*A1PEEPQLRMKNN | 340 4        | 8 612 NIMHSINGYV   | FDSLQ 626     |          | 0 1835 WAYFSDVDLEKDVHS    | 1849   | 92 2121 GTLMVFFGNVDSSGI               | 2135 |
| ŝ            | 53 EFTDHLFNIAKPRPP  | 67  | 27  | 339 NNEEAEDYDDDLTDS    | 353 4        | 19 625 LQLSVCLHEV  | 'AYWYI 639    | 2        | 1 1848 HSGLIGPLLVCHTNT    | 1862   | 93 2134 GIKHNIFNPPIIARY               | 2148 |
| 9            | 66 PPWMGLLGPTIQAEV  | 80  | 28  | 352 DSEMDVVRFDDDNSP    | 366          | 50 638 YILSIGAQTE  | FL.SVF 652    | 14       | 2 1861 NTLNPAHGRQVTVQE    | 1875   | 94 2147 RYIRLHPTHYSIRST               | 2161 |
| 7            | 79 EVYDTVVITLKNMAS  | 69  | 50  | 365 SPSFIQIRSVAKKHP    | 379          | 1 651 VFFSGYTFKF   | KMVYE 665     | 10       | 3 1874 QEFALFFTIFDETKS    | 1888   | <pre>95 2160 STLRMELMGCDLNS*C1C</pre> | 2174 |
| 00           | 92 ASHPVSLHAVGVSYW  | 106 | 30  | 378 HPKTWVHYIAAEEED    | 392          | 52 664 YEDTLTLFPF  | SGETV 678     | 2        | 4 1887 KSWYFTENMERNCRA    | 1901   | 96 2173 SCSMPLGMESKAISD               | 2187 |
| 0            | 105 YWKASEGAEYDDOTS | 119 | 31  | 391 EDWDYAPLVLAPDDR    | 405          | 33 677 TVFMSMENPC  | ILWILG 691    | 2        | 5 1900 RAPCNIQMEDPTFKE    | 1914   | <u>97</u> 2186 SDAQITASSYFTNMF        | 2200 |
| 10           | 118 TSOREKEDDKVFFGG | 132 | 32  | 404 DRSYKSQYLNNGPQR    | 418          | 54 690 LGCHNSDFRN  | RGMTA 704     | 17       | 6 1913 KENYRFHAINGYIMD    | 1927   | 98 2199 MFATWSPSKARLHLQ               | 2213 |
| 12           | 131 GGSHTYVWOVLKENG | 145 | 33  | 417 QRIGRKYKKVRFMAY    | 431          | 55 703 TALLKVSSC*  | AZEITRTT 1654 | 4        | Z 1926 MDTLPGLVMAQDQRI    | 1940   | 99 2212 LQGRSNAWRPQVNNP               | 2226 |
| 12           | 144 NGPMASDPLCLTYSY | 158 | 34  | 430 AYTDETFKTREAIQH    | 444          | 56 1653 TTLQSDQEE1 | DYDDT 1667    | 2        | 1939 RIRWYLLSMGSNENI      | 1953 1 | 00 2225 NPKEWLQVDFQKTMK               | 2239 |
| 13           | 157 SYLSHVDLVKDLNSG | 171 | 35  | 443 QHESGILGPLLYGEV    | 457          | 1666 DTISVEMKKE    | IDFDIY 1680   | 0        | 9 1952 NIHSIHFSGHVFTVR    | 1966 1 | 01 2238 MKVTGVTTQGVKSLL               | 2252 |
| 14           | 170 SGLIGALLVCREGSL | 184 | 36  | 456 EVGDTLLIIFKNQAS    | 470          | 38 1679 IYDEDENQSI | RSFQK 1693    | Ξ<br>Ω   | 1965 VRKKEEYKMALYNLY      | 1979   | 02 2251 LLTSMYVKEFLISSS               | 2265 |
| 15           | 183 SLAKEKTOTLAKFIL | 197 | 37  | 469 ASRPYNIYPHGITDV    | 483          | 39 1692 QKKTRHYFIA | AVERL 1706    | 00       | 1 1978 LYPGVFETVEMLPSK    | 1992   | 03 2264 SSQDGHQWTLFFQNG               | 2278 |
| 16           | 196 TLLEAVEDECKSWHS | 210 | 38  | 482 DVRPLYSRRLPKGVK    | 496 <u>6</u> | 30 1705 RLWDYGMSSS | SPHVLR 1719   | 00       | 2 1991 SKAGIWRVECLIGEH    | 2005 1 | 04 2277 NGKVKVFQGNQDSFT               | 2291 |
| 17           | 209 HSETKNSLMODRDAA | 223 | 6E  | 495 VKHLKDFPILPGEIF    | 509 B        | 11 1718 LRNRAQSGSV | 7PQFKK 1732   | 00       | 2004 EHLHAGMSTLFLVYS      | 2018 1 | 05 2290 FTPVVNSLDPPLLTR               | 2304 |
| <del>2</del> | 222 AASARAWPKMHTUNG | 236 | 4   | 508 IFKYKWTVTVEDGPT    | 522          | 32 1731 KKVVFQEFTI | JGSFTQ 1745   | 80<br>10 | 4 2017 YSN*A3KCQTPLGMASGH | 2031 1 | 06 2303 TRYLRIHPQSWVHQI               | 2317 |
| 6            | 235 NGYVNRSLPGLIGCH | 249 | 4   | 521 PTKSDPRCLTRYYSS    | 535 6        | 33 1744 TQPLYRGELN | IEHLGL 1758   | 8        | 15 2030 GHIRDFQITASGQYG   | 2044 1 | 07 2316 QIALRMEVLGCEAQD*C2            | 2330 |
| 20           | 248 CHRKSVYWHVIGMGT | 262 | 42  | 534 SSFVNMERDLASGLI    | 548          | 54 1757 GLLGPYIRAE | EVEDNI 1771   | -        | 2043 YGQWAPKLARLHYSG      | 2057   |                                       |      |
| 2            | 261 GTTPEVHSIFLEGHT | 275 | 43  | 547 LIGPLLICYKESVDQ    | 561          | 55 1770 NIMUTERNON | SRPYS 1784    | 4        | Z 2056 SGSINAWSTKEPFSW    | 2070   |                                       |      |
| 22           | 274 HTFLVRNHROASLEI | 288 | 44  | 560 DORGNOIMSDKRNVI    | 574          | 56 1783 YSFYSSLISY | TEEDQR 1797   | 7 8      | 2069 SWIKVDLLAPMIIHG      | 2083   |                                       |      |

Totally 107 15-mer peptides moving 13 amino acids were synthesized throughout mature factor VIII polypeptides (amino acid 1-2330) lacking B domain (amino acid 712-1048). \*A1, \*A2, \*A3, \*C1, and \*C2 indicate the carboxyl terminal of each domain. Each peptide has its own number. Position of starting or ending is indicated by the residue number of mature FVIII polypeptide (16).

| Patient | Inhibitor<br>response |                 |                    | Peptide sequences | (FVIII domains)                                 |
|---------|-----------------------|-----------------|--------------------|-------------------|---|
| 1       | Low                   | 1809-1823 (A3), | 1926-1940 (A3),    | 1939-1953 (A3),   | 1965-1979 (A3), 2004-2018 (A3), 2095-2109 (C    |
| 2       | Low                   |                 |                    | -                 |   |
| 3       | High                  | 14-28 (A1),     | 105-119 (A1),      | 1965-1979 (A3),   | 2108-2122 (C1), 2160-2174 (C1), 2316-2330 (C2   |
| 4       | High                  | 144-158 (A1),   | 235-249 (A1),      | 1822-1836 (A3),   | 1848-1862 (A3), 2108-2122 (C1)                  |
| 5       | High                  | 131-145 (A1),   | 1926-1940 (A3),    | 2017-2031 (A3-C   | 1)  |
| 6       | High                  | 248-262 (A1),   | 560-574 (A2),      | 1809-1823 (A3),   | 1926-1940 (A3), 2017-2031 (A3-C1), 2212-2226 (C |
| 7       | High                  | 482-496 (A2),   | 1926-1940 (A3)     |                   |   |
| 8       | High                  | 1926-1940 (A3), | 1939-1953 (A3)     |                   |   |
| 9       | Low                   |                 |                    | -                 |   |
| 10      | Low                   | 1926-1940 (A3), | 2017-2031 (A3-C1), | 2173-2184 (C2),   | 2212-2226 (C2)                                  |
| 11      | High                  | 92-106 (A1),    | 339-353 (A1),      | 1939-1953 (A3),   | 2225-2239 (C2)                                  |
| Normal  |                       | 417-431 (A2).   | 1926-1940 (A3)     | 2004-2018 (A3).   | 2017-2031 (A3-C1)                               |

 Table 3
 Targeted peptide sequences of anti-FVIII antibodies from hemophilia A patients toward B domain-less

 FVIII (A1A2A3C1C2)

Reactive peptide sequence is listed for each hemophilia A patient. Normal indicates pooled plasma from 30 normal male volunteers. FVIII domains in each peptide are indicated with parentheses. Patients 2 and 9 were not analyzed.

2) probe B: p51-61, a 4.7 kb *Eco*RI fragment of FVIII cDNA containing exons 14-26; and 3) probe C: p1.8, a 1.8 kb *Eco*RI fragment containing the remainder of exon 26 from the FVIII gene. The three probes described above were kindly provided by Dr. R. Kaufman at the University of Michigan. Detection of an inversion in the FVIII gene was performed using a "gene a" probe that was a 0.9 kb *Eco*R *I/Sac* I restriction fragment from p482.6 (no.57203; American Type Culture Collection, Rockville, MD, USA) located at intron 22 of the FVIII gene and containing a part of the F8A sequence.

#### RESULTS

#### Epitope mapping of anti-FVIII inhibitor

Eleven hemophilia A patients entered the study but the plasma samples drawn from patients 2 and 9 were not evaluated for the analysis of epitope localization and immunoglobulin subclasses because the inhibitor titers were not high enough for effective analysis (Table 1). Consequently, plasmas from 9 patients were analyzed using 107 peptides covering the functional FVIII amino acid sequences (1-711 and 1649-2330, Table 2).

Fig. 1 demonstrates the results of the ELISA assays and Table 3 summarizes the peptides that were bound by the plasma from 9 patients. In the A2 domain of the heavy chain of human FVIII, plasma from patient 6 showed the major absorbance peak to a peptide covering residues 560-574, and patient 7 to a peptide with residues 482-496. These data indicate that the patients had antibodies recognizing the corresponding residues in the A2 domain (Table 3). Five patients (3, 4, 5, 6, and 11) had antibodies to the A1 domain (Table 3). Consequently, 6 patients had antibodies binding to peptides from the heavy chain of FVIII.

Peptides from the FVIII light chain frequently reacted with hemophilia A patient plasma. Patients 3, 6, 10, and 11 showed binding to peptides in the C2 domain (Table 3). Peptides corresponding to residues 2212-2239 were recognized by 3 patients (6, 10, and 11). Peptides covering 2173-2187 and 2316-2330 were recognized by patients 10 and 3, respectively. In the domain C1, peptides of residues 2095-2122 and 2160-2174 were recognized by 3 patients (1, 3, and 4) and 1 patient (3), respectively.

As compared to the C domains, all the patients' plasma bound to peptides from the A3



Fig. 1-1

Fig. 1 Epitope mapping of anti-FVIII antibodies from hemophilia A patients towards B domain-less FVIII. Nine out of eleven patients were subjected to the FVIII epitope mapping analysis. Patients 2 and 9 were not studied because of low inhibitor titers. Assays were performed using the Pin technology Epitope Scanning Kit described in *Materials and Methods*. Totally, 107 overlapping 15-mer peptides were synthesized on prederivatized polyethylene pins as summarized in Table 2. Epitope study was directly performed for antibody binding to each pin by ELISA as described in *Materials and Methods*. The number along the X-axis denotes the peptide's number listed in Table 2. The Y-axis indicates the observed optic densities (OD) obtained in the ELISA. Each patient plasma specimen as well as normal pooled plasma (N) is denoted in the upper right corner of each panel. All the pins were assayed simultaneously for each plasma specimen to compare the OD values generated. The peptide pins showing values more than the mean ± 2SD were denoted as positively bounded by inhibitor plasma and are whon by the number at the top of the appropriate bar.



Fig. 1-2







Fig. 2A





Fig. 2C



Fig. 2 Detection of gene deletions in inhibitor-positive hemophilia A patients by Southern blotting. Panels A and B show DNA samples from 10 patients digested with *EcoR* I or *Sac* I, respectively, and hybridized with <u>probe A</u>. Panels C and D show the same patient samples as panels A and B digested with *Bam* HI or *Sac* I, respectively, and hybridized with the mixture of <u>probe B</u> and <u>probe C</u>. Numbers above the blot indicate the given patient. A DNA sample for patient 10 was not available. N: DNA from normal control. Characteristics of each patient are shown in Table 1.

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Fig. 3 Detection of gene inversions in inhibitor-positive hemophilia A patients by Southern blotting. DNA from patients 1, 2, 4, 6, and 9 was digested with *Bcl*I, and hybridized with the probe "gene a," corresponding to an intron 22 sequence of the FVIII gene. The number above the blot indicates each patient. DNA samples for patients 4 and 9 exhibited normal patterns, whereas patients 1, 2, and 6 had gene inversions (see "Results" in detail). Characteristics of each patient are shown in Table 1.

domain (Table 3). Two peptide regions covering residues 1809-1834 were recognized by 3 patients (1, 4, and 6). Plasma from 6 patients (1, 5, 6, 7, 8, and 10) shared a peptide 1926-1940 as a target. An overlapping peptide 1939-1953 was recognized by plasma from 3 patients (1, 8, and 11). Patient 1 recognized a peptide 2004-2018.

The two A3 domain peptides 1926-1940 and 2004-2018 were recognized by normal pooled plasma (Table 3). Normal plasma also bound to peptides 2017-2031 in the A3-C1 domain and 417-431 in the A2 domain. It is thus possible that these peptide regions may react to antibody species contained in human plasma.

## Immunoglobulin subclasses of anti-FVIII antibodies

Plasma specimens from the 9 patients were analyzed for immunoglobulin subclasses using the FVIII neutralization method and the results are summarized in Table 1. All the samples had at least a component of IgG4 heavy chain and  $\kappa$  light chain, while IgG2 was uncommon (Table 1). The results are consistent with a previous study which showed both IgG1 and IgG4 antibodies present in 12 inhibitor plasmas.<sup>19)</sup> One hundred percent of the plasma antibody inhibitory activity in patient 3 was dependent on the IgG4 subclass, while both  $\kappa$  and  $\lambda$  chain existed. The light chain type in three plasma specimens from patients 1, 4 and 8 was predominantly  $\kappa$  chain.

## Southern blotting

The results of Southern blotting analysis of DNA samples from 10 patients are shown in Fig. 2 and summarized in Table 1. The genomic DNA sample for patient 10 was not available. Analysis of the hybridization pattern referred to the restriction map for the human FVIII gene published previously.<sup>20,21)</sup> Of our 11 hemophilia A patients with inhibitors, 8 patients (72.7%) had the gross FVIII gene alterations (Table 1).

Gene deletions were identified in 5 patients (3, 5, 7, 8 and 11). The hybridization pattern of probe A that spans exons 1-12, or mixed probes B (exon 14 to the 5' half of exon 26) and C (the 3' half of exon 26), indicated the deletion of exon 1 through 22 in patients 3 and 7 (Fig. 2). Probe A only detected one fragment of *Eco*RI and or *Sac* I from patient 11 (Fig. 2, A and B), whereas the hybridization patterns revealed by probes B and C were the same as those in

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patients 3 and 7 (Fig. 2, C and D). These facts indicate a deletion of exons 2-22 in patient 11. Patient 5 lacked the hybridization of probe A to three *Eco*RI fragments and one *Sac* I fragment, indicating a deletion of exons 2-6 (Fig. 2, A and B). Probes B and C did not hybridize to a 15.0 kb *Bam*HI fragment and a 8.3 kb *Sac* I fragment from patient 8 (Fig. 2, C and D), while the probe A hybridization match of the normal control. Hence, patient 8 appears to lack exons 23-25.

Deletions were not detected in 5 other patients (1, 2, 4, 6 and 9) (Table 1). We then analyzed DNA samples from these patients, digesting each sample with *Bcl*I, then hybridizing with the probe "gene a" corresponding to the intron 22 (Fig. 3). Patients 1 and 2 had the gene inversion between intron 22 and the distal part of the 2 copies of "gene a" located at 500 kb upstream of the FVIII gene. In contrast, patient 6 had a proximal type of inversion (Fig. 3). DNA samples from patients 4 and 9 exhibited a normal pattern (Figs. 2 and 3), indicating they had no large gene abnormalities detectable by Southern blotting analysis.

## DISCUSSION

Epitope mapping scans using the panel of FVIII peptides synthesized on pins, identified a number of reactive sequences both from heavy and light chains (Fig. 1 and Table 3). Unlike the assays based on Western blotting or immunoprecipitation, our assays detect inhibitor epitopes in the absence of protein-denaturing agents. Indeed, our previous study analyzing anti-FIX inhibitors had identified the epitope in 3 hemophilia B patients. These hemophilia B patient epitopes were identical with that of a neutralizing anti-FIX monoclonal antibody, indicating the functional role of the peptide region.<sup>15)</sup>

The A2 domain (residues 380-711) and C2 domain (residues 2176-2332) of human FVIII have been reported to contain major epitopes for anti-FVIII inhibitors.<sup>11-14</sup>) Previous studies had indicated that inhibitors for the C2 domain epitopes interfered with the binding of FVIII to phospholipids, thus appearing to inhibit the FVIII procoagulant activity.<sup>12,22,23</sup> In addition, the stability of FVIII in circulation strongly depends on its binding capacity to VWF via the full C2 domain and the N-terminal region of the A3 domain,<sup>23-25</sup> indicating the importance of the full C2 domain as the inhibitors' target.

Using recombinant hybrid human/porcine FVIII molecules, Healey, *et al.* recently presented residues Glu2181-Val2243 as one of the major determinants of the inhibitory epitope in the C2 domain.<sup>13)</sup> In our study, 4 patients (3, 6, 10, and 11) had antibodies binding to four distinct sequences in domain C2 (Table 3). These peptides were 2173-2187, 2212-2226, 2225-2239, and 2316-2330.

In the heavy chain, patients 6 and 7 had antibodies to the A2 domain residues 560-574 and 482-496, respectively. Healey, *et al.* reported that residues 484-508, corresponding to two of our peptides, 482-496 and 495-509, contained a major epitope in the A2 domain.<sup>11)</sup> An antibody detected in patient 7 is included in this region, although its effect on FVIII activity remains unknown. Fay, *et al.* indicated that residues S558-Q565, corresponding to two of our peptides covering residues 547-574, were important for their association with FIXa.<sup>26)</sup> Therefore, the FVIII-neutralizing activity of plasma from patient 6 is due, in part, to an antibody for the peptide sequence 560-574.

Whereas our findings confirmed the importance of the A2 and C2 domain for the epitope determinant, the A3 domain appears to be another hot target for plasma antibodies (Fig. 1 and Table 3). Plasma from 8 patients bound to a peptide 1926-1940, and 3 patients recognized a neighboring peptide 1939-1953. Another peptide 1809-1821 was recognized by 2 patients.

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Recently, Zhong, *et al.* found that by immunoprecipitation assays, 17 of 18 inhibitor IgGs bound to a recombinant FVIII fragment comprising the A3 and C1 domains, and 3 IgGs prevented binding of FIXa to the FVIII light chain.<sup>27)</sup> The binding was competitively inhibited by their A3 peptide 1804-1819, which overlaps our peptide 1809-1823 (No. 68, Table 2). As well as residues in the A2 domain proposed by Fay, *et al.*,<sup>26)</sup> FIXa binding sites have also been localized in the A3 domain.<sup>28)</sup> Lenting, *et al.* proposed that the region E1811-K1818 is one of the functional FIXa binding sites.<sup>28)</sup> Taken together, it is suggested that antibody species directed to a region by peptide No. 68 (1809-1823) interfere with the binding to FIX, thereby act as anti-FVIII inhibitors.

Not only the patients' plasma but also normal pooled plasma bound to 3 peptides corresponding to residues 1926-1940, 2004-2018, and 2017-2031. Normal plasma was diluted 200 times but patient plasma was diluted 1000-2000 times, suggesting the possibility that the reacted peaks of normal plasma are non-specifically bound.

However, the presence of anti-FVIII antibodies in normal individuals has been reported by several investigators.<sup>29-31)</sup> Interestingly, Gilles, *et al.* showed that 8 out of 10 antibody preparations from healthy individuals inhibited the FVIII binding of their monoclonal antibody recognizing residues 1778-1871.<sup>31)</sup> These findings may suggest that particular regions in the A3 domain present the epitopes of anti-FVIII antibodies without affecting the function of FVIII.

To date, no previous reports have identified the A1 domain as the major inhibitor determinant. However, antibodies to the various portions in the A1 domain were detected in our 5 high responder patients (Table 3). The plasma from all of these patients also recognized other FVIII domains; therefore the functional FVIII inhibition in these patients could be dependent on epitopes other than the A1 domain.

Another study of anti-FVIII inhibitors using immobilized synthetic peptide arrays was performed by Palmer, *et al.*<sup>32)</sup> The length of synthetic peptides (10-mer) was slightly shorter than ours (15 mer) and had only one amino acid overlap. Analysis of 6 hemophilia A patients and 3 non-hemophilia patients with variable inhibitor titers identified several highly reactive peptides throughout the A1-A3 and C1-C2 domains.<sup>32)</sup> Although twenty-two distinct peptides were found reactive to each of 9 patients' plasma, only seven peptides were identical or overlapping with our reactive peptides. In the A1 domain, 3 out of 4 reactive peptides corresponded to our peptides, covering the residues 14-28, 92-106, and 248-262, respectively. Similarly, our four peptides of residues 1965-1979, 2095-2109, 2212-2226, and 2225-2239 from the A3 or C domains were also reactive.<sup>32)</sup> Two peptide regions, 2212-2226 and 2225-2239 have been identified as containing one of the major inhibitor determinants.<sup>13)</sup> However, the other 16 reactive peptides were not included in our list of reactive peptides (Table 3). These facts suggest that the antibody epitopes defined by synthetic peptides may vary with each patient.

Large deletions in the FVIII gene were observed in 5 out of 11 (45.4%) patients. Three patients (27.3%) had an inversion of the FVIII gene (Table 1). Overall, 8 out of 11 patients (72.7%) had either gross deletions or inversions of the FVIII gene, indicating a higher proportion of gross gene alterations in inhibitor-positive hemophilia A patients. Previous multi-center analysis of hemophilia A patients indicated that patients with FVIII gene deletions possess inhibitors to a higher degree (19/60, 31.7%) than patients without gene deletions (8.4%).<sup>8</sup>) Schwaab, *et al.* described a patient group where 43.1% of all patients with inhibitors had deletions, and 34.4% had inversions.<sup>9</sup> Analyzing this information together, it has been suggested that the risk of developing inhibitors is strongly related to stop mutations, large deletions and intrachromosomal recombinations, just like inhibitor-positive patients with hemophilia B.<sup>6,7</sup>

Three patients in our study, with similar partial gene deletions from exon 1 or 2 through 22 (patients 3, 7, and 11), had distinct inhibitor epitopes (Tables 1 and 3). In fact, discordant

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inhibitor development in patients bearing the same FVIII mutation,<sup>21,33)</sup> and in twins or brothers with hemophilia  $A^{34}$  have been described, suggesting the existence of other factors. These factors include HLA<sup>(f)</sup> types<sup>35,36)</sup> or the intensity and duration of replacement therapies.<sup>37)</sup> Genetic prediction of inhibitor epitope specificity would be clinically useful; however, further studies are required to establish the precise relationships between inhibitor targets and defects in the FVIII molecule.

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<sup>&</sup>lt;sup>1)</sup> HLA, Human leukocyte antigen

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