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Monoclonal anti-V_H141 antibodies that specifically recognize the heavy chain variable region of, and are closely related to, MOPC141 myeloma protein whose V_H gene belongs to V_HQ52 family

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SUMMARY

To raise monoclonal antibodies that specifically recognize the heavy chain variable region of MOPC141 myeloma protein (V_H141), which belongs to V_HQ52 family, rats were immunized with Fd′-conjugated keyhole limpet haemocyanin (KLH) (Fd′: Fd′ fragments of MOPC141), and the spleen cells were fused with mouse myeloma cells. The resulting 900 hybridomas were screened for antibody activity against Fd'_1 fragments having no constant H-chain sequences, which were prepared by cleavage of the Fd′ fragments with cyanogen bromide, and two monoclonal antibodies, designated 3-2-7h and 3-5-6f, were obtained. Radioimmunoassay inhibition test showed that the two monoclonal antibodies specifically recognized the V_H141, but each was directed to a different determinant on the V_H141. When the functional V_H gene of Abelson virus-transformed μ-producing pre-B cells, which could be strongly stained with 3-5-6f monoclonal antibody, was cloned and sequenced, the V_H gene was closely relate to that of MOPC141 (88% and 94% homology at amino acid and DNA level, respectively). Taken together, the results indicated that 3-2-7h had high specificity only for the V_H141, whereas 3-5-6f specifically reacted not only with the V_H141 but also with the V_H region closely related to that of MOPC141, and that both the monoclonal anti-V_H141 antibodies were specific for a limited range of V_H regions within the V_HQ52 family rather than being V_HQ52 family specific. These monoclonal anti-V_H141 antibodies should be very useful to determine at a single cell level by immunofluorescence the usage of the V_H gene(s) identical or closely related to that of MOPC141 during early B-cell development.

INTRODUCTION

Immunoglobulin heavy chain variable region genes are completed by two sequential joining events of three gene segments: V_H (variable), D (diversity), and J_H (joining).[1-2] Generally speaking, D to J_H joinings first occur on both chromosomes, followed by V_H to DJ_H joinings during early B-cell development.[3-5] However, V_H to DJ_H joinings on one chromosome occasionally precede completion of DJ_H complexes on both chromosomes, suggesting that these joining events are controlled stochastically, but not strictly in an ordered fashion.[6] V_H replacements,[7-9] secondary D to J_H joinings accompanied with the deletion of the pre-existing DJ_H complexes,[10,11] and V_HDJ_H to J_H joinings also occur.[12]

Murine V_H gene segments are categorized into at least 11 V_H families, based on homology to representative DNA probes.[13] It is thought that utilization of V_H gene segments in variable region formation is non-random.[14-18] However, the mechanism of this non-random usage of V_H gene segments has been poorly understood. The determination at an individual cell level of which V_H family is used in variable region formation is essential and important to elucidate the mechanism. For this purpose we previously raised polyclonal antibodies that specifically reacted with the heavy chain variable region of MOPC315, whose V_H gene belonged to V_H36–60 family, and showed at an individual cell level by immunofluorescence that the usage of the V_H gene(s) identical or closely related to that of MOPC315 was infrequent in nine independent Abelson virus-transformed immature B-cell lines capable of undergoing continuing V_H to DJ_H rearrangements.[18] To gain more insight into the mechanism of V_H gene usage, we wanted to obtain monoclonal antibodies that specifi-

Abbreviations: FITC, fluorescein isothiocyanate; H-chains, heavy chains; KLH, keyhole limpet haemocyanin; L-chains, light chains; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; V_H, variable region of heavy chains.

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cally recognize the heavy chain variable regions of V_H families other than the V_H36–60 family and determine at an individual cell level, by immunofluorescence, the usage of different V_H genes using a panel of antibodies specific for the heavy chain variable regions of different V_H familes. Since the V_HQ52 family was frequently used in some Abelson virus-transformed cell lines, we attempted to prepare the monoclonal anti-V_H antibodies that recognized the heavy chain variable regions of the V_HQ52 family.

In this work we describe the preparation and characterization of monoclonal anti-V_H141 antibodies that specifically recognize the heavy chain variable region of, and closely related to, MOPC141 myeloma protein.

MATERIALS AND METHODS

Myeloma proteins

Myeloma proteins MOPC315 (α, λ_2),[19] MOPC104E (μ, λ_1),[20] J606 (γ3, κ),[21] TEPC15 (α, κ),[22] MOPC141 (γ 2b, κ),[23] MOPC167 (α, κ),[24] and hybridoma MA5-7 (μ, λ_2)[24] were purified from ascites fluids of the respective plasmacytomas, as previously described. Myeloma proteins, MOPC195 (γ2b, κ), and MOPC21 (γ1, κ) were purchased from Organo Technic-Cappel, Westchester, PA.

Preparation of heavy and light chains

Reduction, alkylation and separation of heavy (H) and light (L) chains were performed as described previously.[25] Separated H- and L-chains were dialysed against 5 mM Tris-HCl, pH 8, when cold.

Preparation of Fd′ and Fd′$_1$ fragments from MOPC141 myeloma protein

Purified protein MOPC141 (in 0·1 M Tris-HCl, pH 8·5) was brought to 0·1 M sodium acetate buffer (pH 4·65) by the addition of 0·5 M acetic acid and 0·5 M sodium acetate. To the protein solution, pepsin (10 mg/ml in 0·01 M acetate buffer, pH 4·65) was added to give a weight ratio of 0·65 : 100 of pepsin to the protein MOPC141. After incubation for 4 hr at 37° the digestion was terminated by adjusting the pH to 8·0 with 1 M Tris-HCl, pH 8·6. The solution was applied to a protein A–Sepharose column. After washing with binding buffer (Bio-Rad MAPS-II binding buffer; Hercules, CA), proteins were eluted with elution buffer (Bio-Rad MAPS-II elution buffer). Analysis by SDS–PAGE revealed that F(ab′)$_2$ fragments were contained in the flow-through fractions. Flow-through fractions were collected, dialysed against phosphate-buffered saline (PBS), concentrated, and applied to a Sephacryl S-200 column for further purification.

To prepare Fd′ fragments from F(ab′)$_2$, reduced and alkylated F(ab′)$_2$ fragments (0·01 M DTT and iodoacetamide) were subjected to a DEAE–cellulose column (Whatman DE-52 in 8 M urea/0·05 M Tris-HCl, pH 8·5), and the proteins were eluted with a linear salt gradient of 0–1 M NaCl. Two peaks were obtained, and analysis by SDS–PAGE showed that the first and second peak contained Fd′ fragments and L-chains, respectively. The leading fractions of the first peak were collected, dialysed against 0·2 M NH_4HCO_3, and then lyophilized.

Cyanogen bromide (CNBr) cleavage of Fd′ fragments was performed as follows. Firstly, Fd′ fragments were fully reduced in 6 M guanidine-hydrochloride/0·2 M Tris-HCl, pH 8·5/0·01 M dithiothreitol for 2 hr at room temperature, alkylated with iodoacetamide, and lyophilized after dialysis against 0·2 M NH_4HCO_3. Then the unfolded Fd′ fragments were treated with CNBr (1/5 w/w Fd′ : CNBr) in 70% formic acid for 4 hr at room temperature and lyophilized after termination of the reaction by adding 10 volumes of distilled water.

Since Fd′ fragments have two methionine residues at positions 82 and 187, they are cleaved to yield three peptides, i.e. Fd′$_1$, Fd′$_2$ and Fd′$_3$ fragments (named from N-terminus).[19] The CNBr-treated Fd′ was chromatographed on a DEAE–cellulose column; firstly with loading buffer (8 M urea/0·05 M Tris-HCl, pH 8·5), and then the column was eluted with elution buffer (8 M urea/0·05 M Tris-HCl, pH 8·5/0·3 M NaCl). Based on SDS–PAGE analysis, the flow-through fraction contained Fd′$_1$ fragments, as expected from known amino acid sequence data that Fd′$_1$, Fd′$_2$ and Fd′$_3$ fragments are basic, neutral, and acidic peptides, respectively. To purify further the Fd$_1$′ fragments, the flow-through fractions were subjected to gel filtration on Sephadex G-50 column in 6 M guanidine hydrochloride/0·2 M NH_4HCO_3, and the fractions containing Fd′$_1$ fragments were collected, dialysed, and lyophilized, and the highly purified materials were used as antigen for the final screening of hybridomas producing anti-V_H141 antibodies.

Preparation of monoclonal anti-V_H141 antibodies

Female SD rats were immunized subcutaneously by injecting 0·3 mg of KLH-conjugated Fd′ fragments emulsified in complete Freund's adjuvant followed by the second subcutaneous immunization with 0·3 mg of KLH-conjugated Fd′ fragments in incomplete Freund's adjuvant 2 weeks later.[26] Two weeks after the second injection, intraperitoneal booster injection of 0·1 mg of the conjugate in PBS was daily performed for two consecutive days. Three days after the injection, spleen cells were fused with P3-X63-Ag.8.653 cells. Culture supernatants of hybridomas were firstly screened for antibody activity against the H-chains of MOPC141 myeloma protein by an enzyme-linked immunosorbent assay (ELISA). Hybridomas with antibody activity for the H-chains were secondarily screened for antibody activity for the purified Fd′$_1$ fragments by ELISA. Hybridomas that produced antibodies against the Fd′$_1$ fragments were tested further by ELISA using a panel of different purified antigens.

Iodination and radioimmunoassay

Monoclonal antibodies were isolated by Sepharose 4B on which H-chains of MOPC141 had been conjugated. Affinity-purified monoclonal anti-V_H141 antibodies were labelled with ^{125}I (Amersham, Amersham, Bucks, U.K.) by chloramine-T method. Specific activities of the ^{125}I-labelled antibodies were -5×10^6 c.p.m./μg antibody proteins. The specificity of the monoclonal anti-V_H141 antibodies was examined by a solid-phase direct binding radioimmunoassay. Microtitre plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with H-chains of MOPC141 (20 μg/ml, 100 μl/well) by overnight incubation at 4°. After washing and saturating free sites with 1% bovine serum albumin/BBS-Az, ^{125}I-labelled anti-V_H141 antibodies (10 ng/25 μl/well) and various concentrations of inhibitors (0·01 to 200 μg/ml, 100 μl/well) were added to the wells in triplicate and incubated at 4° overnight. After washing, wells were counted in a gamma counter.

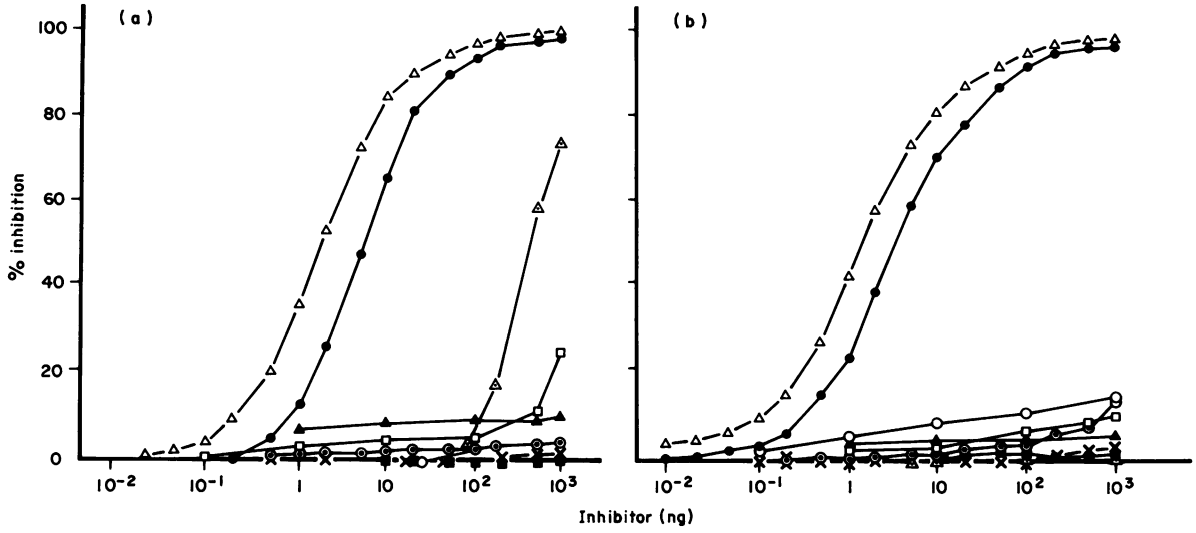

Figure 1. Inhibition radioimmunoassay of the binding of monoclonal anti-V_H141 antibodies to the H-chains of MOPC141 (HM141) by various H-chains and intact MOPC141 IgG molecules. The ability of different H-chains and intact MOPC141 IgG molecules to inhibit the binding of monoclonal anti-V_H141 antibody 3-2-7h (A) or 3-5-6f (B) to HM141 was determined as described in the Materials and Methods. The inhibitors used were: Fd'_1 (△); H-chains of MOPC141 (●), TEPC15 (⊙), MOPC195 (○), MOPC21 (□), J606 (■), MOPC104E (▲), MOPC315 (×), MOPC141 IgG (▲).

Immunofluorescence

Cells fixed with acid-ethanol were double-stained with FITC-conjugated monoclonal anti-V_H141 antibodies plus rhodamine-conjugated anti-mouse μ-chain antibodies, and examined by a fluorescent microscope.

DNA cloning and sequencing analysis of V_H gene

The functional V_HDJ_H allele of AT8-1-12-5-6-2 pre-B cells double-stained by monoclonal anti-V_H141 antibody 3-5-6f plus anti-μ antibodies was cloned as J_H-associated XbaI fragments in λ ong C. Appropriate restriction fragments were ligated into PUC19 vector and sequenced by dideoxy methods.

RESULTS

Isolation of hybridomas producing monoclonal antibodies against the Fd'_1 fragments of MOPC141 myeloma protein

SD rats were hyperimmunized with the Fd'-conjugated KLH, and hybridomas were produced. When the culture supernatants from 900 hybridoma-growing wells were screened for antibody activity against H-chains of MOPC141 myeloma protein (HM141), 30 wells gave positive. Culture supernatants from 30 wells were rescreened for antibody activity against Fd'_1 fragments having no constant H-chain sequence, and only five wells showed positive for the V_H determinants. Then hybridomas from the five wells were cloned twice by limiting dilution in ORIGEN Hybridoma Cloning Factor (IGEN Inc., Rockville, MD) at 10% (v/v) and further characterized.

To determine precisely the antibody activity of the monoclonal antibodies against HM141, binding assay was performed. Five monoclonal antibodies were affinity purified from culture fluid supernatants using HM141–Sepharose 4B. The purified antibodies from the five hybridomas were labelled with ^{125}I, and 20 ng of the ^{125}I-labelled antibodies were added to microtitre plates pre-coated with HM141(20 μg/ml, 100 μl/well). After incubation at 4° overnight, the plates were intensively washed with PBS, and the radioactivities of the wells were counted. In two hybridomas, named 3-2-7h and 3-5-6f, 42.5% and 42·8% of the ^{125}I-labelled antibodies added to the wells bound to the wells, respectively. In the remaining three hybridomas, only 8–10% of the input radioactivity reacted with antigen on the wells. Therefore, we chose the two hybridomas 3-2-7h and 3-5-6f for the further detailed characterization.

Fine specificity of monoclonal anti-V_H141 antibodies 3-2-7h and 3-5-6f

To determine the specificity of the two monoclonal anti-V_H141 antibodies 3-2-7h and 3-5-6f, we examined the inhibition of binding of ^{125}I-labelled monoclonal anti-V_H141 antibodies to HM141 by various inhibitors (Fig. 1). Fd'_1 fragments completely inhibited the binding of the two monoclonal anti-V_H141 antibodies to HM141. HM141 was a weaker inhibitor than Fd'_1 fragments, but 100% inhibition was obtained at a higher concentration of HM141. The inhibition of binding of the monoclonal anti-V_H141 antibodies to HM141 by intact IgG molecules of MOPC141 was observed at a high concentration of the IgG molecules in 3-2-7h, but not in 3-5-6f. When various H-chains from MOPC104E, TEPC15, MOPC315, MOPC21, J606, and MOPC195 were used as inhibitors, no H-chains significantly inhibited the binding of the two monoclonal anti-V_H141 antibodies to HM141. Furthermore, when inhibition radioimmunoassay was performed using L-chains of MOPC141, MOPC167, hybridoma MA5-7, MOPC2020, and MOPC315 as inhibitors, only L-chains of MOPC141 weakly inhibited the binding of 3-2-7h to HM141 at a high concentration (Fig. 2). Thus, both 3-2-7h and 3-5-6f showed very high specificity for the binding to Fd'_1 fragments although 3-2-7h weakly reacted with intact IgG molecules of MOPC141 at high concentration (compared to HM141, an approximately 100-fold higher concentration of intact IgG molecules was required for 50% inhibition).

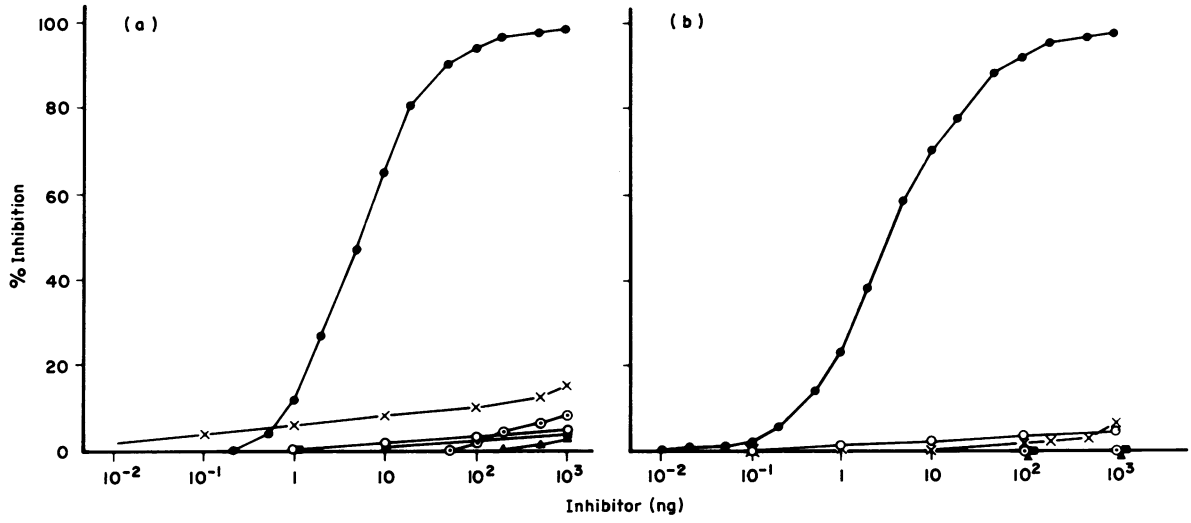

Figure 2. Inhibition radioimmunoassay of the binding of monoclonal anti-V_H141 antibodies to the H-chains of MOPC141 by various L-chains. Percentage inhibition of the binding of monoclonal anti-V_H141 antibody 3-2-7h (a) or 3-5-6f (b) to HM141 by the indicated L-chains was measured as described in the Materials and Methods. The inhibitors used were: H-chains of MOPC141 (●); L-chains of MOPC141 (×), MOPC167 (○), MOPC202 (⊙), MOPC315 (■); and hybridoma MA5-7 (▲).

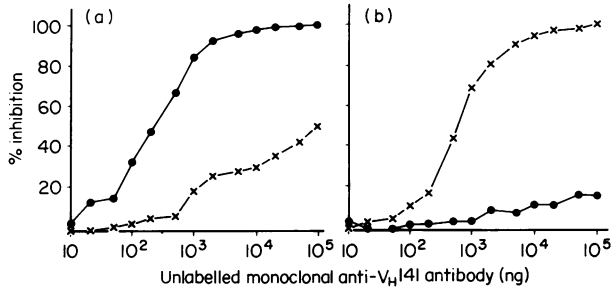

Figure 3. Inhibition radioimmunoassay of the binding of ^{125}I-labelled monoclonal anti-V_H141 antibodies to the H-chains of MOPC141 by unlabelled monoclonal anti-V_H141 antibodies. Percentage inhibition of the binding of ^{125}I-labelled monoclonal anti-V_H141 antibody 3-2-7h (a) or 3-5-6f (b) to HM141 by unlabelled 3-2-7h (●) and 3-5-6f (×) was measured as described in the Materials and Methods.

Furthermore, the results suggested that the two monoclonal anti-V_H141 antibodies 3-2-7h and 3-5-6f recognized different epitopes on the heavy chain variable region of MOPC141; therefore, inhibition of the binding of ^{125}I-labelled monoclonal anti-V_H141 antibodies to HM141 by unlabelled monoclonal anti-V_H141 antibodies was measured (Fig. 3). The binding of ^{125}I-labelled 3-2-7h to HM141 was completely inhibited by 10^4 ng of unlabelled 3-2-7h, whereas the binding was inhibited only 50% by 10^5 ng of unlabelled 3-5-6f. On the other hand, almost complete inhibition of the binding of ^{125}I-labelled 3-5-6f to HM141 was attained by 10^4 ng of unlabelled 3-5-6f, whereas only 15% inhibition was found by 10^5 ng of unlabelled 3-2-7h. Thus, these results indicated again that 3-2-7h and 3-5-6f presumably recognized different epitopes on the heavy chain variable region of MOPC141.

The heavy chain variable region of μ-producing pre-B cells stained strongly with monoclonal anti-V_H141 antibody 3–5–6f is encoded by the V_H gene closely related to that of MOPC141

Next, we determined the specificity of the monoclonal anti-V_H141 antibodies at cellular level. When MOPC141 myeloma cells were stained with FITC-conjugated monoclonal anti-V_H141 antibodies 3-2-7h and 3-5-6f, the cells were strongly stained with both the antibodies. When an Abelson virus-transformed cell line AT8-1-12-5-6, which is capable of generating μ-positive pre-B cells via *in vitro* continuing IgH gene rearrangements, was stained with the FITC-conjugated monoclonal anti-V_H141 antibodies, approximately 0·01–0·1% of the cells was positively stained with 3-5-6f, but none of the cells was stained with 3-2-7h. To characterize further these positively stained cells, cell cloning was performed, and the AT8-1-12-5-6-2 subclone, where all the cells were positive for staining with 3-5-6f, was isolated. Next, the functional V_H gene of AT8-1-12-5-6-2 was cloned and sequenced (Fig. 4). The V_H gene of AT8-1-12-5-6-2 had 88% and 94% homology with the V_H gene of MOPC141 at amino acid and DNA level, respectively. Thus, the results clearly demonstrated that 3-5-6f could recognize the heavy chain variable regions identical or closely related to that of MOPC141 at cellular level. As expected, 3-2-7h could not stain this AT8-1-12-5-6-2 subclone. Furthermore, when Abelson virus-transformed μ-positive pre-B cells AT11-2-5-1-5-51-1 (whose V_H gene belongs to V_HQ52 family, refs 3, 4, and 17) and AT8-1-4 (whose V_H belongs to V_H30–60 family) were stained with both the FITC-conjugated monoclonal anti-V_H141 antibodies, neither AT11-2-5-1-5-51-1 nor AT8-1-4 was stained with these antibodies. Taken together, these results indicated that 3-2-7h had high specificity probably only for the heavy chain variable region of MOPC141, whereas 3-5-6f specifically reacted not only with the heavy chain variable region of MOPC141 but also with the heavy chain variable region closely related to that of MOPC141, and that both the monoclonal anti-V_H141 antibodies were specific for a limited range of heavy chain variable regions within the V_HQ52 family rather than being V_HQ52 family specific.

DISCUSSION

Initially we tried to prepare hyridomas from spleen B cells of rats immunized with Fd′ fragments alone without conjugating them

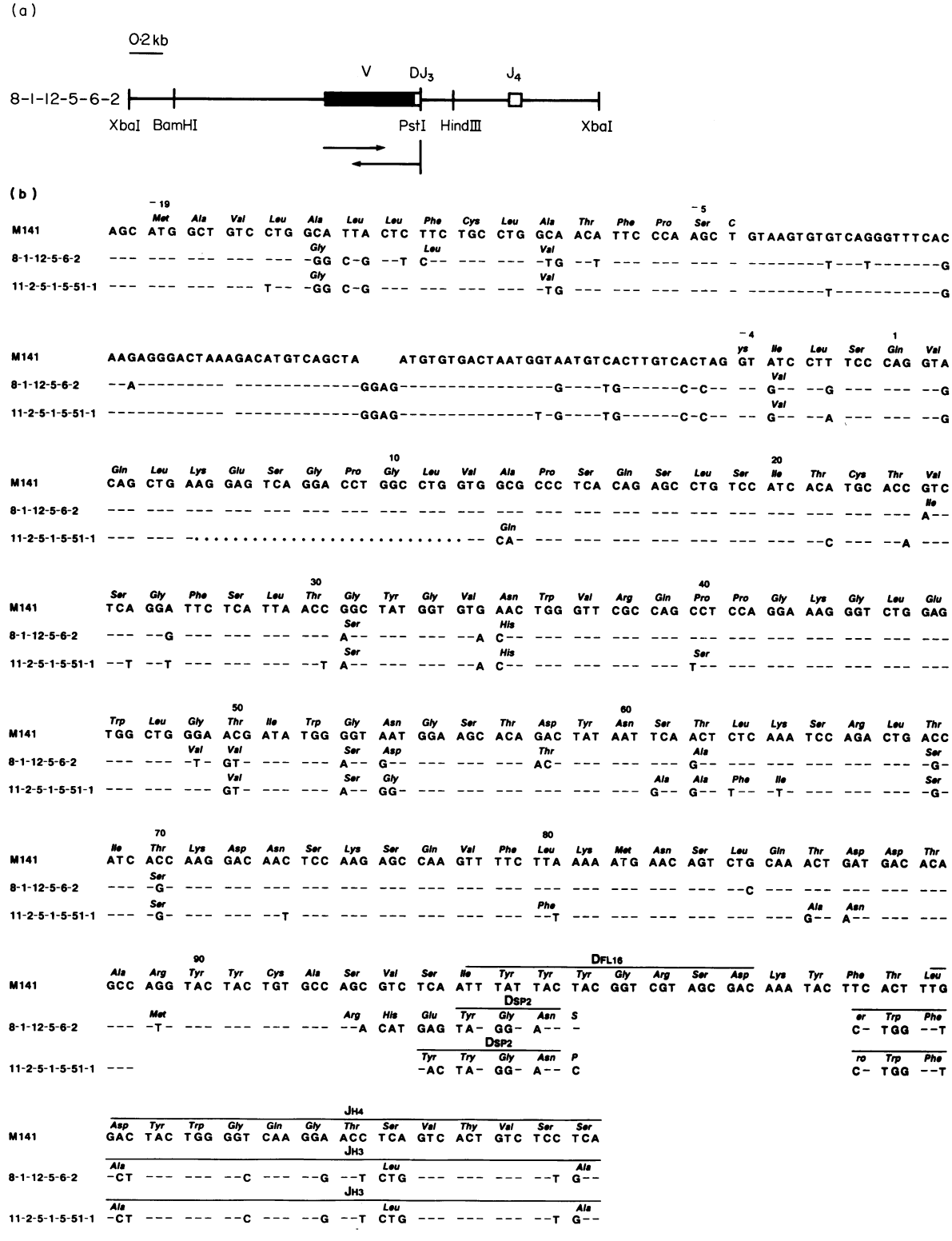

Figure 4. Nucleotide and amino acid sequence of the heavy chain variable region recognized by monoclonal anti-V_H141 antibody 3-5-6f. (a) Restriction maps of the cloned $V_HDJ_H^+$ allele of AT8-1-12-5-6-2 μ-positive pre-B cells stained with FITC-conjugated monoclonal anti-V_H141 antibody 3-5-6f. (b) Nucleotide and amino acid sequence of the $V_HDJ_H^+$ allele of AT8-1-12-5-6-2. Amino acid and nucleotide sequences of MOPC141[2] and AT11-2-5-1-5-51-1[3,4,17] are described for comparison. Dashes indicate identity with reference sequence.

to a carrier protein KLH. However, by this immunogen, animals did not produce any detectable antibodies to the heavy chain variable region of MOPC141 myeloma protein. To overcome this problem, we attempted to immunize rats with Fd′ fragments which had been covalently coupled to KLH with the aid of glutaraldehyde, and indeed we have succeeded in producing monoclonal anti-V_H141 antibodies directed to the heavy chain variable region of MOPC141 myeloma protein.

Theoretically, monoclonal anti-V_H antibodies could be classified into three types based on the antigenic determinants on the heavy chain variable regions to which the monoclonal antibodies (mAb) were directed: (i) mAb directed to idiotypic determinants on the heavy chain variable regions, (ii) mAb which recognize framework portions, and (iii) mAb which recognize V_H family-specific antigens. Since both the monoclonal anti-V_H141 antibodies, 3-2-7h and 3-5-6f, did not react with different H-chains other than HM141, it seems that these monoclonal anti-V_H141 antibodies do not recognize framework portions. Among three cell lines examined, MOPC141, AT8-1-12-5-6-2, and AT11-2-5-1-5-51-1, whose V_H genes belonged to the same V_HQ52 family, 3-2-7h reacted with only one cell line MOPC141, and 3-5-6f reacted with two cell lines, MOPC141 and AT8-1-12-5-6-2. This indicated that neither 3-2-7h nor 3-5-6f was V_HQ52 family specific. It would be reasonable to conclude that 3-2-7h was directed to an idiotypic determinant expressed on the heavy chain variable region of MOPC141 myeloma protein and 3-5-6f was directed to a cross-reactive idiotypic determinant expressed on a limited range of heavy chain variable regions of V_HQ52 family. In a related study, by immunization of AKR mice with purified V_H315 fragments, two sets of monoclonal anti-V_H antibodies, one set specific to a V_H subgroup and the other set demonstrating a wider range of specificity toward many H-chains from various species, were prepared.[27] However, in the present studies we could not get any monoclonal anti-V_H antibodies with a wider range of specificity. Whether we could prepare only monoclonal anti-V_H141 antibodies with a much restricted range of specificity as a matter of course based on the procedures used here, or by chance, is of interest, but cannot be resolved at present. Immunizations of other animals, including hamsters, with Fd′-KLH, followed by establishment of anti-V_H monoclonal antibodies, are planned.

Since 3-5-6f reacted with only isolated H-chains, but not with intact form, of MOPC141 myeloma protein, the monoclonal antibody seemed to recognize a 'hidden' determinant which was not exposed on intact Ig molecules. On the other hand, since 3-2-7h reacted weakly with intact MOPC141 IgG molecules, it seemed to recognize a determinant which was exposed weakly on the intact Ig molecules and more strongly on the isolated H-chains. Monoclonal anti-V_H antibodies raised against purified V_H315 fragments also recognized only isolated H-chains.[27] Furthermore, monoclonal anti-V_H antibodies raised against isolated Vμ fragments of human immunoglobulins also recognized only isolated H-chains, but not the intact molecules.[28] 3-5-6f and these monoclonal anti-V_H antibodies against V_H315 or Vμ fragments that reacted with only isolated H-chains might recognize sequential determinants expressed in the primary protein structure. The reason why 3-5-6f reacts only with AT8-1-12-5-6-2 between the two pre-B cell lines AT8-1-12-5-6-2 and AT11-2-5-1-5-51-1, although their V_H regions are closely related, is unknown. Compared with AT11-2-5-1-5-51-1, AT8-1-12-5-6-2 has higher homology in the V_H region to MOPC141 (88% versus 81% at amino acid sequence, and 94% versus 89% at DNA sequence). This might be one of the plausible reasons. Using synthetic peptides, analysis of a determinant recognized by 3-5-6f would be needed.

With the advent of our presently established valuable monoclonal anti-V_H141 antibodies, we are currently carrying out experiments to estimate how often the heavy chain variable regions identical or closely related to those of MOPC141 were used in V_H to DJ_H joinings in many different pre-B-cell lines capable of undergoing continuing V_H to DJ_H rearrangements. These monoclonal anti-V_H141 antibodies should be very useful for fine analysis of V_H gene usage at an individual cell level during early B-cell development.

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