IMMUNOGLOBULIN V_H DETERMINANTS DEFINED BY MONOCLONAL ANTIBODIES*

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The heavy and light chains of immunoglobulins (Ig) consist of two distinct regions: variable and constant. Variable regions, which determine the antibody specificity and idiotypic determinants, are further divided into three hypervariable regions and four relatively invariant, so-called framework, regions (1, 2). The variable regions of both heavy chains (V_H)[1] and light chains (V_L) have been divided into subgroups based upon similarities between amino acid sequences in their framework regions. For human Ig, three major V_H subgroups have been characterized on the basis of amino acid sequences of the aminoterminal 20 residues within the first framework region (2). One set of goat antibodies specific for each human V_H subgroup has been prepared by selective adsorption and was found to be useful in typing secreted and membrane-bound Ig on B cells (3).

Evidence from studies (4–8) using anti-idiotype antibodies has suggested that T cells and their antigen-specific, soluble factors might possess Ig-like idiotypic determinants. V_H allotypic determinants have also been demonstrable on rabbit T cells by several groups (9, 10) and not by others (11–15). Antibodies against V_H framework regions of the mouse myeloma protein MOPC-315, which cross-react with other mouse Ig regardless of their antibody specificity, class, subclass, subgroup, or allotype (16), may inhibit T cell function (17–19) and cross-react with soluble factors of T cells (20, 21). The molecular nature of antigen receptors on T lymphocytes, however, is still controversial. Recent studies (22, 23) using recombinant DNA techniques have suggested that antigen-specific helper or killer T cell clones may not use the Ig-joining and constant gene segments.

We prepared monoclonal hybridoma antibodies against V_H fragments isolated from human IgM myeloma proteins with the hope of using these to analyze the products of normal or abnormal B and T cells. This paper describes the preparation and characterization of four such monoclonal anti-human V_H antibodies. Our studies

[1] *Abbreviations used in this paper:* anti-C_H, antibodies to heavy chain isotype; anti-C_L, antibodies to light chain type; anti-Id, antibodies to idiotypic determinants; AP, alkaline phosphatase; BSB, borate saline buffer; cIg, cytoplasmic immunoglobulins; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HAT, hypoxanthine-aminopterin-thymidine; NTCB, 2-nitro-5-thiocyanobenzoic acid; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; RITC, rhodamine isothiocyanate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sIg, surface immunoglobulins; V_H, variable portions of immunoglobulin heavy chains; V_L, variable portions of immunoglobulin light chains; $V\mu$, variable portions of μ heavy chains.

show that monoclonal V_H-specific antibodies are more readily prepared using isolated V_H fragments as immunogens and that these antibodies are specific for "hidden" determinants not exposed on intact Ig molecules.

Materials and Methods

Mice. Inbred BALB/c and (BALB/c × C57BL/6)F$_1$ hybrid (CB6) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and used at 2–3 mo of age.

Isolation of Vμ Fragments from Human IgM Paraproteins. IgM paraproteins were isolated from the sera of patients with Waldenström's macroglobulinemia or multiple myeloma by repeated euglobulin precipitation against distilled water, followed by gel filtration on a Sepharose 6B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) column equilibrated with borate saline buffer, pH 8.3, ionic strength, 0.1 (BSB). The elution peak containing pentameric IgM was pooled, concentrated, and dialyzed against 0.1 M Tris-HCl buffer, pH 8.6, containing 2 mM EDTA. Some of the IgM paraproteins with known V_H and V_L subgroups were kindly provided by Dr. J. D. Capra (University of Texas, Dallas, TX). The purity of isolated IgM was tested by immunoelectrophoresis and double diffusion in agar, using antisera or antibodies specific for normal whole serum proteins, IgM, IgG, and IgA.

Vμ fragments were isolated from IgM paraproteins by a modification of methods described by Rodwell and Karush (24). The amount of 2-nitro-5-thiocyanobenzoic acid (NTCB; Eastman Kodak Co., Rochester, NY) for cyanylation of the sulfhydryl groups of reduced IgM was used at a ratio of NTCB/IgM of 5 mg/100 mg. This ratio corresponded approximately to a five-fold molar excess of NTCB to protein-associated sulfhydryl groups. NTCB was used in this study without purification, although its melting point was 2–3°C lower than the reported value (25). The reaction mixture containing the cleavage products was lyophilized, dissolved in 3 ml of 5 M guanidine-HCl, pH 7.0, containing 2 mM EDTA, and then applied to a calibrated Sephadex G-150 column equilibrated with the same denaturant. The fractions containing Vμ fragments, which eluted after the light chain peak, were collected, dialyzed against BSB to remove guanidine, and then purified further by passage through a Sepharose 4B column to which the homologous light chains were coupled (26). The bound Vμ fragments were eluted from the column by 0.05 M glycine-HCl buffer, pH 2.8, neutralized with BSB, and then dialyzed against saline before use for immunization.

IgG, IgA, and IgD myeloma proteins were also isolated by salt fractionation, DEAE ion-exchange cellulose column chromatography, and Sepharose 6B gel filtration, as described elsewhere (27). IgEλ myeloma protein (PS, V_{HIII}, $V_{\lambda III}$) was a gift from Dr. K. Ishizaka (Johns Hopkins University, Baltimore, MD). Heavy and light chains were isolated by partial reduction and alkylation of purified myeloma Ig or pooled normal IgG followed by separation on Sephadex G-200 in 5 M guanidine-HCl, pH 7.0. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slabs of 5–20% acrylamide gradient gels in Tris-HCl buffer, as described by Laemmli (28).

Immunization. BALB/c and CB6 mice were immunized 6–7 times with 80–100 μg of purified Vμ fragments, isolated μ chains, or intact myeloma proteins at 3- or 7-d intervals. In some cases, mice were immunized with Vμ fragments at 14-d intervals for the first four injections, rested for 2 mo, and then immunized again at 7-d intervals for the last three injections. The first injection was always given with complete Freund's adjuvant (Difco Laboratories, Detroit, MI), the second in incomplete Freund's adjuvant, and the remainder in saline. All mice were immunized subcutaneously in footpads, inguinal, and axillary regions. Mice were killed 1 d after the last immunization, and the cells from the draining popliteal, inguinal, axillary, and para-aortic lymph nodes were prepared by gentle teasing and used for fusion.

Preparation of Monoclonal Anti-V_H Antibodies. Lymph node cells from immunized mice were fused with an equal number of P3-X63-Ag.8.653 cells, a non-Ig-producing variant of the hypoxanthine-aminopterin-thymidine (HAT)-sensitive BALB/c myeloma cell line P3-X63-Ag.8, by using polyethylene glycol 4000 (Fisher Scientific Company, Atlanta, GA), as described previously (29, 30). The cells were plated into 24-well plates (Costar, Data Packaging, Cambridge, MA) at a cell concentration of 2 × 10^5 cells/ml in the HAT medium. 2–3 wk after fusion, culture supernatants from the wells were screened for antibody activity against the

various immunogens by an enzyme-linked immunosorbent assay (ELISA) (31). Affinity-purified goat anti-mouse κ and λ antibodies labeled with alkaline phosphatase (AP) were used as second-step reagents (31). After detecting hybrid-containing wells with antibody activities, the antibody specificities were tested further by ELISA against a panel of 8–12 different purified antigens, including V_μ fragments, various intact Ig, isolated heavy or light chains, and $F(ab')_2$ fragments of normal IgG. Hybridomas that secreted antibodies reacting with common or homologous V_H determinants in this initial screening were then chosen for further study and recloned by limiting dilution on syngeneic normal mouse peritoneal exudate feeder cells. The Ig isotypes of the hybridoma antibodies were also determined at this stage using AP-labeled goat antibodies specific for mouse μ, γ_1, γ_{2a}, γ_{2b}, γ_3, α, κ, and λ chain determinants, as described elsewhere (32).

10–30 million cells from positive reclones were injected intraperitoneally into syngeneic mice previously primed with pristane (Aldrich, Milwaukee WI). Ascites were harvested, and monoclonal anti-V_H antibodies were purified from the ascites fluid by absorption to Sepharose 4B coupled with the appropriate antigens. Bound antibodies were eluted from the column by 0.05 M glycine-HCl buffer, pH 2.8. In some experiments, antibodies precipitated from hybridoma ascites by 60% saturation of ammonium sulfate were used as the anti-V_H antibody preparation after dialysis against BSB.

Radioimmunoassay. The specificity of monoclonal anti-V_H antibodies was examined by using a solid-phase, direct binding radioimmunoassay. 96-well polyvinyl plastic plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with anti-V_H antibodies (10 μg/ml) by overnight incubation at room temperature. After washing and saturating free sites on the plastic plates with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in BSB, various concentrations of purified antigens, including intact Ig and isolated heavy or light chains, were added to the antibody-coated wells in triplicate and incubated for 4 h at 37°C. After washing out unbound antigens, ^{125}I-labeled monoclonal hybridoma antibodies specific for human μ, γ, κ, or λ determinants ($\sim 10^5$ cpm/100 μl) were added to each well and incubated for 4 h at 37°C. Unbound radioactivity was washed out with BSB, and the wells were counted in a gamma counter. All monoclonal hybridoma antibodies used for ^{125}I-labeling were purified by affinity chromatography.

Cell Preparation. Peripheral blood lymphocytes (PBL) were isolated from normal adults by Ficoll-Hypaque density gradient centrifugation. Bone marrow specimens were obtained with informed consent from normal volunteers or patients with monoclonal gammopathies by aspiration and with parental consent from fetuses aborted for reasons of maternal health by suction curettage. Mononuclear cells were isolated from bone marrow samples by previously described methods (33). Gestational age was estimated by measuring long bone length (34). Normal tonsillar and splenic tissues were dispersed into a single cell suspension by gentle teasing in phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS; Flow Laboratories Inc., Rockville, MD.). The use of these materials for research purposes was approved by the University of Alabama in Birmingham committee on human experimentation. After washing with PBS containing 5% FCS, the cells were resuspended in RPMI 1640 supplemented with 20% FCS and incubated in plastic plates for 1 h at 37°C to remove adherent cells and cytophilic IgG. T cells were enriched from PBL by rosette formation with 2-aminoethyl-isothiouronium bromide (AET; Sigma Chemical Co.)-treated sheep erythrocytes and density gradient separation (35). In some experiments, PBL or enriched T cells were cultured in the presence of phytohemagglutinin M (PHA, 5 μl/ml) at a cell concentration of 5 × 10^5 cells/ml of RPMI 1640 containing 20% FCS for 2 d at 37°C under 5% CO_2.

Immunofluorescent Analysis of Cells. The characteristics of V_H^+ cells were determined by two-color immunofluorescence as previously described (27, 33). For surface staining, 10^6 viable cells were incubated with 10 μl of monoclonal anti-V_H antibodies (0.5 mg/ml) for 20 min at 4°C, washed, and stained with 10 μl of rhodamine-isothiocyanate (RITC)-labeled $F(ab')_2\gamma$ fragments of rabbit anti-mouse Ig antibodies (0.4 mg/ml; P/R ratio, 2.1). After washing out free conjugates, the cells were counterstained with 10 μl of fluorescein isothiocyanate (FITC)-labeled goat anti-human Ig antibodies (0.5 mg/ml; F/P ratio, 3.5). FITC-labeled goat antibodies specific for human Ig isotype (μ, γ, α, κ) were also used for counterstaining. After washing, the doubly stained cells were cytocentrifuged onto glass slides, fixed in 95% ethanol-5% acetic acid

at −20°C, rehydrated in PBS, and then mounted in Elvanol. Smears of unstained cells were similarly prepared by cytocentrifugation, fixed, and stained with one-fifth the concentration of the same reagents. Both affinity-purified antibodies for labeling with RITC (anti-mouse Ig) or FITC (anti-human Ig or anti-isotype) were extensively absorbed with normal pooled human Ig and human IgM myeloma proteins or with normal pooled mouse Ig and mouse IgM myeloma proteins, respectively. Pre-B cells, B lymphocytes, and plasma cells expressing V_H determinants were identified as previously described (27, 33).

Results

Purification of Vμ Fragments by Affinity Chromatography. Vμ fragments were isolated from human IgM myeloma proteins by the following steps: (a) reduction of interdisulfide bonds of IgM molecules with dithiothreitol, (b) cyanylation of their sulfhydryl groups with NTCB, and (c) cleavage of IgM molecules at the amino side of the cyanylated residues by alkaline pH, as described by Rodwell and Karush (24). The isolation profile of such a reaction mixture, as determined by Sephadex G-150 gel filtration in 5 M guanidine-HCl, was essentially the same as their reported profile on Sephacryl S-200 gel filtration. The Vμ peak, however, contained a few additional protein fragments that migrated between the light chain and Vμ bands on SDS-PAGE (Fig. 1). These protein fragments (or side products) did not decrease in quantity, even after reduction of the molar ratio of NTCB/IgM from a 25- to a 5-fold excess (data not shown). Subsequent cleavage was done at a five-fold excess.

When the Vμ fragments containing side products from a Sephadex G-150 column were applied to a Sepharose 4B column coupled with the homologous light chains, only the Vμ fragments bound to the light chain column. The elution of Vμ fragments by acid buffer afforded highly purified Vμ fragments (Fig. 1, lane 1).

These findings indicate that Vμ fragments isolated by selective cyanylation and cleavage can bind to the homologous light chains under physiological conditions, and this provides an efficient method of purifying isolated Vμ fragments.

Production of Monoclonal Anti-V_H Antibodies. In our initial attempts to prepare monoclonal antibodies to common V_H determinants, we immunized mice with intact Ig molecules or isolated heavy chains. The culture supernatants from 6–24% of wells,
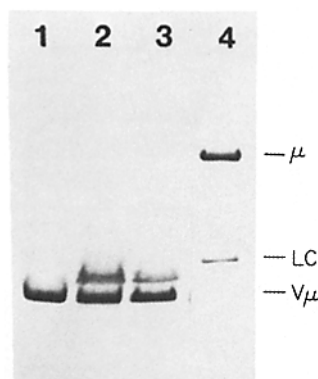
Fig. 1. SDS-PAGE of isolated Vμ fragments by affinity chromatography using the homologous light chain coupled column. Vμ samples isolated from Sephadex G-150 gel filtration (lane 3) were applied onto Sepharose 4B coupled with the homologous light chains. The bound Vμ fragments were eluted from the column by acid buffer (lane 1). Lane 2 is the fraction of Vμ samples that failed to bind to the column. Lane 4 shows the marker proteins, μ heavy chain and κ light chain.

containing lymph node cells derived hybridomas from mice immunized with intact IgMκ (SA, V_{HI}, and Vκ, untyped), IgDλ (010), or IgEλ (PS, V_{HIII}, $V_{\lambda III}$), showed antibody activity against the immunizing antigens (Table I). 40–70% of these positive wells contained antibody specific for the heavy chain isotype of the immunogen (anti-C_H); i.e., anti-μ, anti-δ, or anti-ϵ. About 10–30% had antibody specific for the light chain type of the homologous immunogen (anti-C_L), i.e., anti-κ or anti-λ. The remaining positive wells produced antibodies reactive only with the homologous intact myeloma proteins, probably anti-idiotype (anti-Id). None of the monoclonal antibodies prepared in this way were reactive with isolated Vμ fragments or with multiple heavy chain isotypes. Similarly, the culture supernatants of hybridomas derived from mice immunized with the μ chain (007, V_{HIII}) showed mostly anti-μ activities, except for one well that reacted only with the homologous intact myeloma protein; no anti-V_H antibodies were detected.

In contrast to the above results, when hybridomas were derived from mice immunized with isolated Vμ fragments, 6–12% of wells contained hybridomas producing antibodies against the Vμ immunogen, the parent IgM molecules, or isolated μ chains. About 30–50% of these positive wells showed antibody activities to V_H determinants, 30–50% to μ chains, 0–10% to light chains, and 10–50% to idiotypic determinants. Two hybrid-containing wells were untyped; one of them, resulting from the fusion of Vμ (SA) immunized cells, produced antibody reactive with all myeloma proteins tested as well as bovine serum albumin-coated plates, and the other, formed by fusion of Vμ (NF) immunized cells, reacted with the homologous intact IgM and one of seven unrelated myeloma proteins. The latter antibody brightly stained the cytoplasm of all normal bone marrow cells, including myeloid cells, as determined by immunofluorescence.

Among 11 anti-V_H wells from the fusion of Vμ (NF)-immunized cells, 6 reacted with several Vμ fragments, including homologous Vμ fragments, one with the homologous Vμ fragment only, and the other four with homologous Vμ, several intact myeloma proteins, and F(ab')$_2$ fragments of normal IgG. Three anti-V_H wells from the fusion of Vμ (MH)-immunized cells and eight anti-V_H wells from the fusion of Vμ (SA)-immunized cells showed a similar spectrum of specificities within each group. Even though Vμ fragments purified by elution from an affinity column bearing homologous light chains were used as immunogens, some of the fused cells from

TABLE I

Specificity of Monoclonal Anti-Immunoglobulin Antibodies

Immunogen	Number of wells positive/total*	Number of wells with the following specificities‡				
		Anti-C_H	Anti-C_L	Anti-Id	Anti-V_H	Untyped
IgMκ (SA)	42/192	23	13	6	0	0
IgDλ (010)	34/144	23	4	7	0	0
IgEλ (PS)	26/432	10	3	13	0	0
μ (007)	30/168	29	0	1	0	0
Vμ (MH)	10/150	5	1	1	3	0
Vμ (SA)	16/264	5	0	2	8	1
Vμ (NF)	27/220	8	3	4	11	1

* Positive wells contained a single heavy chain and light chain isotype and antibody activity against the immunogen.

‡ Specificity was determined by ELISA using plastic plates coated with 8–12 different antigens.

immunized mice produced antibodies against light chains.

These findings indicate that, at least in our hands, hybridomas secreting anti-V_H antibodies can be efficiently produced by the fusion of cells from mice immunized with isolated $V\mu$ fragments, but not with intact Ig molecules or isolated heavy chains.

Fine Specificity of Monoclonal Anti-V_H Antibodies. Four representative monoclonal anti-V_H antibodies were prepared by recloning hybridoma clones in limiting dilution and used in this study. MH-44, NF-11, and SA-44 were derived from the fusion of cells from mice immunized with $V\mu$ (MH), $V\mu$ (NF), and $V\mu$ (SA), respectively (see Table I). GB-24 was derived from the fusion of cells immunized by $V\mu$ (GB) (not shown in Table I). The MH-44 and GB-24 monoclonal antibodies were $\mu\kappa$ in isotype, and NF-11 and SA-44 were $\gamma_1\kappa$ antibodies.

To determine the V_H specificity of these monoclonal antibodies, we conducted the following radioimmunoassay. Various concentrations of homologous intact IgM or isolated μ or light chains were added to wells precoated with monoclonal anti-V_H antibodies. ^{125}I-labeled monoclonal anti-human μ or ^{125}I-labeled mixtures of monoclonal antibodies to human κ and λ chains were used to measure the bound IgM or μ chains or the bound light chains, respectively. As shown in Fig. 2, all four monoclonal anti-V_H antibodies reacted well with homologous μ chains, but minimally with intact homologous IgM molecules and light chain preparations. MH-44 appeared to be reactive with the homologous IgMκ (MH) at the highest concentration in this experiment. This preparation of IgMκ (MH) had been stored at 4°C for several months and might have undergone a degree of spontaneous degradation. Indeed, in another experiment, freshly thawed IgMκ (MH) preparations from storage at -20°C showed no reactivity with MH-44 over the same range of concentrations (data not shown). The observation that the anti-V_H antibodies failed to react significantly with the homologous intact IgM and light chains was not because of binding properties of the second antibodies used in the above assay system because (a) monoclonal anti-human μ antibodies used for iodination bound to intact IgM molecules as well as to the isolated μ chains, and the same was true for a mixture of monoclonal anti-κ and anti-λ antibodies, and (b) the amounts of both ^{125}I-labeled anti-μ and ^{125}I-labeled anti-light chain reagents to be added were adjusted to give the same cpm to the wells precoated with intact IgM molecules. Furthermore, the wells precoated with monoclonal anti-V_H antibodies (10 μg/ml) exhibited <1% binding of ^{125}I-labeled homologous intact myeloma Ig, whereas the wells coated with monoclonal anti-Id antibodies (10 μg/ml) derived from the same fusion showed 60–70% binding.

Similarly, all four anti-V_H antibodies reacted with pooled normal γ chains at different degrees but did not react with the intact IgG molecules or light chains (data not shown).

Table II shows the reactivity of these anti-V_H antibodies against a panel of human myeloma heavy chains as determined by ELISA on antigen-coated plates. This assay system was usually used for screening the hybridoma clones secreting anti-V_H antibodies. Their V_H reaction spectra overlapped but were distinctive. MH-44 reacted with one of two V_{HI} myeloma and one V_{HII} myeloma heavy chains but not with four V_{HIII} myeloma heavy chains tested. GB-24 reacted with one of two V_{HI} myeloma and four V_{HIII} myeloma proteins, but not with a V_{HII} myeloma protein. Both NF-11 and SA-44 were reactive with one of two V_{HI} myeloma and two of four V_{HIII} myeloma heavy chains.
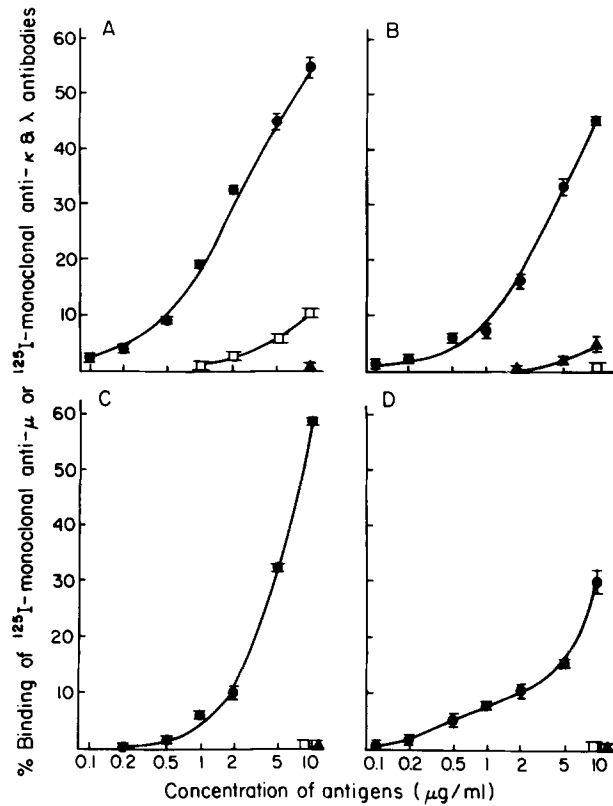
FIG. 2. Direct binding radioimmunoassay of monoclonal anti-V_H antibodies to their homologous immunoglobulin components. The plates were precoated with the following monoclonal anti-V_H antibodies (10 μg/ml). (A) MH-44, (B) GB-24, (C) NF-11, and (D) SA-44. Various concentrations of their homologous intact IgM (□), homologous μ chains (●), or homologous light chains (▲) were added. The bound IgM or μ chains were measured by ^{125}I-labeled monoclonal anti-human μ antibodies, and the bound light chains were measured by ^{125}I-labeled mixture of monoclonal antibodies to human κ and λ determinants.

Similar results were obtained by immunofluorescent staining for the plasma cells from patients with monoclonal gammopathies (multiple myeloma, Waldenström's macroglobulinemia, benign monoclonal gammopathy, and light chain disease) (Table III). Among 20 cases of monoclonal gammopathies, excluding light chain disease, MH-44 anti-V_H antibodies were reactive with 8 cases (2μ, 1δ, 3γ, 2α), GB-24 with 5 cases (1μ, 1δ, 3γ), NF-11 with 5 cases (2μ, 2γ, 1α), and SA-44 with 3 cases (3μ). None of the affected cells from individuals with light chain disease were V_H^+.

Taken together, these results suggest that (a) these anti-V_H antibodies recognize V_H determinant(s) only and not combinatorial determinants of V_H and V_L portions of the Ig molecules; (b) the V_H reaction spectra for a panel of myeloma Ig show overlapping but distinctive patterns for the four antibodies, indicating that each sees a different V_H determinant; and (c) these monoclonal antibodies recognize common V_H determinant(s), the distribution of which does not conform to the conventional V_H subgroup assignment.

Immunofluorescent Analysis of the Expression of V_H Determinants by Normal Cells of B and T

TABLE II

Reactivity of Monoclonal Anti-V_H Antibodies, as Determined by ELISA, Using Myeloma Heavy Chain-coated Plates

Donor	Heavy chain antigen	V_H subgroup	Monoclonal anti-V_H antibodies			
			MH-44	GB-24	NF-11	SA-44
GA	μ	I	+	+	+	+
001	$\alpha 1$	I	−	−	−	−
003	$\alpha 1$	II	+	−	−	−
SA	μ	III	−	+	+	+
007	μ	III	−	+	+	+
McK	μ	III	−	+	−	−
PS	ϵ	III	−	+	−	−
MH	μ	ND*	+	+	−	−
GB	μ	ND	+	+	−	+
NF	μ	ND	−	−	+	+
DOS	μ	ND	−	−	−	+
010	δ	ND	+	+	−	−
MG	γ	ND	+	+	+	−

* Not determined.

Lineages. To examine whether normal human plasma cells, lymphocytes, and pre-B cells may express the determinants recognized by these monoclonal anti-V_H antibodies, indirect immunofluorescence was used. Substantial proportions of normal plasma cells from bone marrow, spleen, and tonsils were stained for their cytoplasmic Ig (cIg) with the anti-V_H antibodies (Table IV). MH-44, GB-24, and NF-11 stained 24–34%, 14–24%, and 13% of the total cIg$^+$ plasma cells, respectively. SA-44 showed less reactivity with normal plasma cells (~3%). A pool of the four monoclonal anti-V_H antibodies reacted with ~55% of the total plasma cells, whereas the sum of each V_H^+ frequency was 67%, suggesting overlapping V_H reactivities with these antibodies. All V_H^+ plasma cells were also stained with polyvalent anti-human Ig antibodies, indicating that these anti-V_H antibodies did not react with non-Ig components.

The reactivity of the anti-V_H antibodies with normal plasma cells was not restricted to any particular heavy chain class or light chain type (Table V). However, NF-11 and SA-44 appeared to react with more μ^+ plasma cells than γ^+ and α^+ plasma cells, whereas MH-44 and GB-24 tended to react with higher proportions of the IgA plasma cells.

When viable lymphocytes obtained from normal peripheral blood were stained with anti-V_H antibodies, both surface Ig-bearing (sIg$^+$) B cells and sIg− T cells were shown to be unreactive (Table IV). The failure to stain sIg on viable B cells with anti-V_H antibodies was consistent with the radioimmunoassay results (Fig. 2), indicating that the anti-V_H antibodies did not bind intact Ig molecules. On the other hand, the anti-V_H antibodies were reactive with surface Ig molecules after air drying and acid-alcohol fixation of the cells. The frequencies of B cell subpopulations reactive with the four monoclonal anti-V_H antibodies were very similar to those observed for plasma cells. By contrast, none of the anti-V_H antibodies were reactive by immunofluorescence with T cell constituents either before or after cell fixation and with or without PHA stimulation.

The monoclonal anti-V_H antibodies were reactive with the intracytoplasmic μ chain

TABLE III

*V$_H$ Reactivity to Plasma Cells from Patients with Monoclonal Gammopathies,
as Determined by Indirect Immunofluorescence*[*]

Patient	Monoclonal isotype	Plasma cells	Percent V$_H$$^+$ cells among the indicated plasma cells			
			MH-44	GB-24	NF-11	SA-44
HW	IgMκ	μ^+	1	<1	<1	<1
007	IgMκ	μ^+	<1	95	100	95
LK	IgMκ	μ^+	96	<1	<2	98
SM	IgMλ	μ^+	86	<1	16	<1
GL	IgMλ	μ^+	<1	<1	92	89
010	IgDλ	δ^+	100	100	<1	<1
MAH	IgG$_{1\kappa}$	γ^+	1	<1	<1	<1
IS	IgG$_{1\kappa}$	γ^+	3	<1	1	<1
WAT	IgG$_{1\kappa}$	γ^+	100	<1	99	<1
VL	IgG$_{1\kappa}$	γ^+	<1	95	<1	<1
TAY	IgG$_{1\lambda}$	γ^+	100	97	93	<1
JH	IgG$_{2\kappa}$	γ^+	<1	<1	<1	<1
015	IgG$_{4\lambda}$	γ^+	100	97	<1	<1
001	IgA$_{1\kappa}$	α^+	<1	<1	<1	<1
003	IgA$_{1\lambda}$	α^+	90	<2	5	<2
WC	IgA$_{1\lambda}$	α^+	99	1	97	<1
JN	IgA$_{1\lambda}$	α^+	<1	<1	<1	<1
AB	IgA$_{1\lambda}$	α^+	<1	<1	<1	<1
313	IgA$_{1\lambda}$	α^+	<1	<1	<1	<1
014	IgA$_{2\kappa}$	α^+	<1	<1	<1	<1
BRD	κ	κ^+	1	<1	<1	<1
OJ	κ	κ^+	1	<1	1	<1
IRW	κ	κ^+	<1	1	<1	<1
DS	κ	κ^+	3	<1	<1	<1
PR	λ	λ^+	<1	<1	<1	<1

[*] Fixed bone marrow or blood plasma cells were stained for cytoplasmic Ig, as
described in Materials and Methods.

of pre-B cells in bone marrow samples from fetuses (Table IV). The frequencies of
V$_H$$^+$ pre-B cells were three- to fivefold less than those of V$_H$$^+$ plasma cells, except with
the SA-44 antibody.

These results indicate that the monoclonal anti-V$_H$ antibodies can be used to detect
heterogeneity of plasma cells, B cells, and pre-B cells, but the intact surface Ig
molecules on viable B cells are not recognized by these antibodies without prior
denaturation. V$_H$ determinants could not be detected at all by immunofluorescence
analysis of either resting or activated T cells.

Discussion

To prepare antibodies against common V$_H$ determinants of human Ig, we used
hybridoma-derived antibodies rather than conventional heteroantisera. When intact
Ig molecules or isolated heavy chains were used as immunogens, we did not obtain
antibodies that were reactive with Vμ fragments or multiple heavy chain isotypes.
Monoclonal anti-V$_H$ antibodies could be readily obtained, however, by using isolated
Vμ fragments as immunogens. Four monoclonal anti-V$_H$ antibodies, MH-44 ($\mu\kappa$), GB-

Table IV

Expression of V_H Determinants by Normal Human Plasma Cells, Lymphocytes, and Pre-B Cells, as Determined by Indirect Immunofluorescence

Cell phenotype and source	Percent cells reactive with monoclonal anti-V_H antibodies*			
	MH-44	GB-24	NF-11	SA-44
cIg$^+$ plasma cells				
Bone marrow (N = 9)‡	34 ± 11	14 ± 5	13 ± 6	3 ± 2
Spleen (N = 6)	33 ± 6	14 ± 3	13 ± 8	2 ± 1
Tonsil (N = 4)	23 ± 2	24 ± 8	13 ± 5	4 ± 3
sIg$^+$ B cells				
Blood, viable (N = 5)	≤1.4	<0.1	<0.1	<0.1
Blood, fixed (N = 5)	38 ± 7	13 ± 3	22 ± 9	6 ± 1
Cμ^+ pre-B cells				
Fetal bone marrow§ (N = 6)	10 ± 7	3 ± 2	4 ± 4	2 ± 1
T cells ‖				
Blood, unstimulated (N = 5)	<0.02	<0.02	<0.02	<0.02
Blood, PHA-stimulated (N = 3)	<0.05	<0.05	<0.05	<0.05

* The results are recorded as the mean ± 1 SD.
‡ The number of specimens is given in parethesis.
§ 14–17 wk of gestation.
‖ T cells were stained before and after acid-alchol fixation in an attempt to detect either surface or cytoplasmic V_H determinants.

Table V

Expression of V_H Determinants by Each Class of Plasma Cells in Normal Bone Marrow Samples*

Antibody	Percent V_H^+ plasma cells among			
	IgM$^+$	IgG$^+$	IgA$^+$	κ^+
MH-44	27 ± 5	26 ± 4	36 ± 8	35 ± 6
GB-24	15 ± 2	15 ± 7	22 ± 8	18 ± 10
NF-11	18 ± 9	8 ± 5	7 ± 2	14 ± 5
SA-44	10 ± 6	2 ± 1	5 ± 1	3 ± 1

* The results indicate mean ± 1 SD from five normal marrow samples.

24 ($\mu\kappa$), NF-11 ($\gamma_1\kappa$), and SA-44 ($\gamma_1\kappa$), were prepared from different fusions. The evidence that these antibodies are directed toward V_H, rather than C_H, V_L, or C_L determinants, is as follows: (a) they react with their homologous $V\mu$ fragments and isolated μ heavy chains but not with the intact homologous IgM molecules or light chains, (b) they also react with pooled normal γ chains but not with the intact IgG molecules or light chains, and (c) their reactivities are not restricted to particular heavy chain classes. The isolated $V\mu$ fragments are known to contain about 15 additional amino-terminal residues of the $C\mu_1$ domain (24). However, the restriction of the reactivity of the anti-V_H antibodies to certain μ chains and the fact that they react with normal γ chains and isolated heavy chains of other isotypes rules out the possibility that these antibodies recognize $C\mu_1$ determinants. Moreover V_H^+ plasma cells in normal bone marrow samples were found among all major isotypes (μ, γ, α, κ) of plasma cells, as determined by immunofluorescence. These data suggest that the four monoclonal antibodies are indeed directed toward the V_H determinant(s).

All four of the monoclonal anti-V_H antibodies reacted with isolated heavy chains

but not with intact, undenatured Ig molecules, a finding anticipated from the ease of producing heavy and light chain-specific antibodies directed against determinants that are not exposed on the intact molecule (36). Procedures that might be expected to cause mild denaturation exposed the "hidden" V_H determinants, e.g., adherence of the myeloma protein to a plastic surface was usually sufficient to allow reactivity with the homologous anti-V_H antibody that could be detected by an enzyme-linked immunosorbent assay (data not shown). Similarly, the anti-V_H antibodies did not bind membrane-bound Ig molecules on viable B cells. However, when blood mononuclear cells were pretreated with an acid-alcohol fixing solution, subpopulations of B cells were detectable by immunofluorescence staining with the monoclonal anti-V_H antibodies (37). The frequencies of the V_H^+ B cell subpopulations (38% MH-44$^+$, 13% GB-24$^+$, 22% NF-11$^+$, 6% SA-44$^+$) were in close agreement with the relative frequencies of the different subpopulations of V_H^+ plasma cell. Indirect immunofluorescence could also be used to detect subpopulations of human pre-B cells that contain cytoplasmic μ heavy chains only. Thus, the anti-V_H antibodies can be used to identify subpopulations of B lineage cells at all stages in differentiation during which they express heavy chains.

The reactivity of the monoclonal anti-V_H antibodies with isolated heavy chains of known V_H subgroups did not appear to correspond to the conventional V_H subgroup typing. The V_H reaction spectra with a panel of myeloma heavy chains and with plasma cells from monoclonal gammopathy patients revealed overlapping but distinctive patterns for the four antibodies (Tables II and III). 3–35% of normal bone marrow plasma cells were stained by immunofluorescence with the individual anti-V_H antibodies, and, when combined, they stained >50% of normal plasma cells. Although human V_H allotypes have been reported (38, 39), these anti-V_H antibodies are apparently not allotype-specific for the following reasons: (a) plasma cells from over 20 individuals, including Caucasians, Blacks, and Orientals, were stained with the anti-V_H antibodies in similar frequencies, and (b) the monoclonal anti-human V_H antibodies were also reactive with Ig heavy chains of other mammals, including mouse (manuscript in preparation). These findings, thus, suggest that our anti-V_H antibodies recognize highly conserved but not subgroup-specific V_H determinants and that each antibody recognizes a different V_H epitope.

Our monoclonal anti-V_H antibodies, thus, differ in specificity from the V_H subgroup-specific antibodies reported by Natvig and his colleagues (3). The latter antibodies were raised in goats or rabbits by immunizing with intact myeloma proteins with known V_H and V_L subgroups. Three sets of antibodies prepared by selective adsorption were rendered specific for each human V_H subgroup without overlapping reaction, and these preparations reacted with intact Ig molecules as well as isolated heavy chains. We were unable to obtain monoclonal antibodies specific for the described human V_H subgroups during the course of this study, suggesting that there might be a very limited number of unique determinants on human Ig that can elicit V_H subgroup-specific antibodies in the mouse. It is possible that a mixture of different monoclonal anti-V_H antibodies, each of which recognizes small populations of Ig molecules, might behave as V_H subgroup-specific reagents analogous to the anti-V_H subgroup antisera.

Other investigators have also attempted to prepare heterologous antisera against V_H framework determinants of mouse (16, 40) and human (41–45) Ig. These antisera

were raised in rabbits (16, 40–43, 45) or chickens (44) by immunizing with Fv (16) or Fab (40) fragments of mouse myeloma proteins and isolated heavy chains (41), V_H fragments (42, 44, 45), or intact molecules (43) of human myelomas. After appropriate absorptions, these antisera were rendered specific for idiotypic determinants (V_H-V_L), including cross-reactive idiotypes, and V_H framework determinants, suggesting that these contain mixtures of antibodies with different specificities toward V_H or V_H plus V_L. All of these antisera, with one exception (16), reacted with intact Ig molecules better than with isolated heavy chains. One antiserum, anti-V_H 315 (16), exhibited similar reactivity to ours, except for the fact that it also contained anti-idiotypic antibodies; our anti-V_H antibodies did not recognize exposed determinants, even on the homologous myeloma molecules. Recently, rabbit antisera against similarly prepared mouse $V\mu$ fragments have been characterized, and their reactivities appeared to be similar to our monoclonal anti-V_H antibodies, except for their reactivity with intact Ig molecules.[2]

There is considerable evidence (46, 47) that T cells and their products may express idiotypic or "framework" V_H determinants, suggesting that V_H gene products in T cells may play an important role in antigen recognition (46, 47). It has also been reported that (a) the anti-V_H 315 antibodies (16) inhibit T cell function in mice (17–19) and cross-react with antigen-specific, soluble factors of T cells (20, 21) (b) unabsorbed chicken antisera against human $V\mu$ fragments stain by immunofluorescence ~30% of normal T cells (44), and (c) unabsorbed rabbit antibodies against the V_H fragments of a human $IgG_{3\kappa}$ myeloma also show weak reactivity by immunofluorescence with ~20% of normal T cells and may inhibit mixed lymphocyte reactions (45). In contrast, our monoclonal anti-V_H antibodies appear unreactive by immunofluorescence with either surface or cytoplasmic components of both resting and activated T cells, and this is consistent with the results of others using goat anti-V_H subgroup antibodies (48), rabbit anti-V_H framework antibodies (42), and rabbit V_H allotype antibodies (11–15). Our monoclonal anti-V_H subset antibodies could be valuable reagents in the future analysis of this issue using very sensitive assays of antigen-specific, soluble factors of murine and human T cells. They also provide useful probes to analyze the expression of V_H determinants produced by normal and abnormal B cells (37).

Summary

Hybridoma clones secreting antibodies against common V_H determinants were readily produced by fusion of cells from mice immunized with isolated $V\mu$ fragments of human immunoglobulins (Ig), but not with intact Ig molecules or isolated heavy chains. Four monoclonal antibodies to the $V\mu$ fragments of different IgM paraproteins were selected for analysis: MH-44 ($\mu\kappa$), GB-24 ($\mu\kappa$), NF-11 ($\gamma_1\kappa$), and SA-44 ($\gamma_1\kappa$). Each antibody reacted with the homologous $V\mu$ fragment, homologous μ chain, and normal γ chains, but not with the intact IgM molecules, intact IgG, or isolated light chains, as determined by radioimmunoassay. The V_H reaction spectra with a panel of myeloma heavy chains showed overlapping but distinctive patterns for the four antibodies. Each of the four monoclonal anti-V_H antibodies appeared to react with a different "hidden" V_H determinant that is not exposed on undenatured, intact Ig

[2] Karjalainen, K. The preparation of mouse V_H fragments and the characterization of heterologous anti-mouse V_H antibodies. Manuscript submitted for publication.

molecules and differs from conventional V_H subgroup determinants. In immunofluorescence studies, the monoclonal anti-V_H antibodies did not bind to surface Ig on viable B lymphocytes, but visibly stained subpopulations of fixed B lymphocytes, pre-B cells, and normal plasma cells. The mean frequencies of V_H^+ plasma cells were 30% (MH-44), 17% (GB-24), 13% (NF-11), and 3% (SA-44), and similar frequencies were obtained for the V_H^+ B cell subpopulations. While subpopulations of B cells could be identified at all stages in differentiation by immunofluorescence with the anti-V_H antibodies, neither resting nor activated T cells expressed these V_H determinants in detectable amounts.

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