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Monoclonal antibodies reactive with idiotypic and variable-region specific determinants on human immunoglobulins

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Summary. Monoclonal antibody JG-B1, specific for the human V_KIIIb sub-subgroup of L chains, and JG-B4 specific for an idiotypic determinant on Glo, a monoclonal human IgM-V_KIIIb anti-IgG, were produced and characterized. The V_KIIIb determinant was detected on L chains alone and intact immunoglobulins with V_KIIIb L chains. However, the idiotypic determinant was expressed only on IgM-Glo and required association of H and L chains. Binding of the immunogen Glo, to its antigen-IgG partially inhibited anti-idiotype and anti-V_KIIIb binding. Cross-inhibition experiments demonstrated that intact pentameric IgM-Glo expressed one-half the number of idiotypic sites as V_KIIIb determinants. However, Glo half-molecules expressed equal numbers of idiotypic and V_KIIIb determinants. This is the first described monoclonal antibody produced by hybridoma technology which recognizes an antigenic determinant specific for a single variable region in intact immunoglobulin.

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Abbreviations: CAF_1, $(BALB/c \times A/J)F_1$ mice; ELISA, enzyme-linked immunoassay; H, heavy chain; Ig, immunoglobulin; L, light chain; RIA, radioimmunoassay; AP, alkaline phosphatase.

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INTRODUCTION

Human autoantibodies with antigenic specificity for IgG have related antigenic and molecular structures. These include cross-reactive idiotypes (Kunkel *et al.*, 1973; Førre *et al.*, 1979; Agnello *et al.*, 1980), light and heavy chain variable region subgroups (Kunkel *et al.*, 1974; Førre, Johnson & Natvig, 1977; Johnston, Abraham & Welch, 1975), and a high degree of primary amino acid sequence homology of heavy and light chains (Franklin & Frangione, 1971a; Capra & Kehoe, 1974; Capra & Klapper, 1976). Many of these shared properties have been demonstrated by study of anti-IgG autoantibodies (rheumatoid factors) obtained from patients with mixed cryoglobulinaemia whose serum contains a monoclonal cryoprecipitable IgM anti-IgG.

Structural and serological studies show that the majority of these IgM cryoproteins have type-K light chains of the V_KIII subgroup (Kunkel *et al.*, 1974; Johnston *et al.*, 1975; Wang, Wells & Fudenberg, 1974; Capra & Kehoe, 1974), and the V_KIIIb sub-subgroup (Kunkel *et al.*, 1974; Capra & Kehoe, 1975; Ledford *et al.*, 1982; Feizi, Kunkel & Roelcke, 1974). This apparent restriction has interesting implications since autoantibodies of other specificities also contain light chains which belong to the same subgroup and sub-subgroup (Feizi *et al.*, 1976; Kunkel, Joslin & Hurley, 1976).

Mixed IgM–IgG cryoglobulins and some other human autoantibodies have been divided by anti-idiotypic reagents into cross-reactive idiotype groups (Kunkel *et al.*, 1973; Førre *et al.*, 1979; Capra & Kehoe, 1974; Kunkel *et al.*, 1976; Feizi *et al.*, 1974). While the fine specificities of the anti-idiotypic antisera used in these studies has not been defined, one cross-reactive idiotype group of mixed cryoglobulins all contained light chains of the V_KIIIb sub-subgroup (Kunkel *et al.*, 1974). These studies have all been performed utilizing polyclonal rabbit antisera in which numerous antigenic specificities have initially been present and precise determination of the relationships between the unique idiotype and subgroup specific antigens has not been possible. In instances, the polyclonal antisera used to define the cross-reactive idiotypes of these mixed cryoglobulins, were apparently not specifically absorbed with immunoglobulins which contained only V_KIIIb light chains. Thus, it may be difficult to conclude what the fine specificity of these anti-idiotypic reagents were.

It is for these reasons that we have begun to define idiotypic and variable region markers on human autoantibodies with monoclonal reagents. While production of monoclonal antibodies utilizing hybridoma techniques is now a common technique, monoclonal antibodies which selectively react with individual human heavy or light chain variable region subgroup antigens have been difficult to produce and detect (Kubagawa *et al.*, 1982).

The present study describes two murine monoclonal reagents, JG-B1 and JG-B4. JG-B1 was shown to be specific for V_KIIIb light chains present in either intact human immunoglobulin (Ig) molecules or free light chains. JG-B4 was specific for an idiotypic determinant on the IgM V_KIIIb anti-IgG cryoglobulin, Glo. The idiotype was detected only on intact Glo and not on isolated heavy or light chains. The effect of antigen (i.e. IgG) binding on the expression of the V_KIIIb and idiotypic determinants, and the ability of JG-B1 or JG-B4 to competitively inhibit binding of the other to IgM-Glo was also determined.

MATERIALS AND METHODS

Mice

(BALB/c × A/J)F_1 (CAF_1) female mice were purchased from the Jackson Laboratory, Bar Harbor, ME.

Proteins used

Monoclonal IgM anti-IgG cryoglobulins were isolated from serum or plasma of patients with mixed cryoglobulinemia by precipitation at 4°, washing of the cryoglobulin in chilled buffer and purification by DE-52 chromatography at 37° as previously described (Johnston *et al.*, 1975). Glo plasma was the generous gift of Dr J. Vaughan (Scripps Clinic and Research Foundation, La Jolla, CA). The purity of the IgM cryoglobulins and their IgG antigens was shown by SDS-PAGE, and by immunodiffusion and immunoelectrophoresis in agar gel utilizing antisera specific for heavy chain class and light chain type determinants.

The various myelomas and Bence-Jones proteins used to test the specificity of the monoclonal antibodies have been described previously (Johnston *et al.*, 1975; Johnston & Abraham, 1979; Solomon & McLaughlin, 1969; McLaughlin & Solomon, 1972; Abraham, Clark & Vaughan, 1972).

Reduction and alkylation of All (IgG1-K) and Glo (IgM-K) was performed by standard techniques (Fleishman, Pain & Porter, 1962) in order to obtain purified heavy and light chains of each protein. In addition, Glo was mildly reduced and alkylated (Scoville, Abraham & Turner, 1979) under non-dissociating conditions to obtain half molecules, as determined by SDS-PAGE run under non-reducing conditions. IgG-All Fab was prepared by papain digestion, as previously described (Johnston & Abraham, 1979).

Media

Hybridoma and parent-cell lines were cultured in a modified Mishell-Dutton medium (Greenstein *et al.*, 1981), supplemented with 10% foetal calf serum and 2X sodium pyruvate (Gibco, Grand Island, NY).

Production of hybridomas

Monoclonal antibody specific for the variable region of the mixed cryoglobulin Glo was produced by a modification of the protocol of Köhler & Milstein (1975). A CAF_1 mouse was first injected intraperitoneally with a tolerizing dose (2·5 mg) of a deaggregated human IgM-V_KIII (Hoe), as described for human IgG by Chiller & Weigle (1971). Ten days later the mouse was primed with 150 μg of glutaraldehyde (1%) aggregated (Avrameas & Ternyck, 1969) IgM-Glo in Freund's complete adjuvant. After 15 days, a boosting dose of 75 μg of native IgM-Glo was injected, and 3 days later the spleen was removed and dissociated for fusion. The spleen cells were washed and fused with 50% polyethylene glycol 4000 (MC/B

Manufacturing Chemists, Cincinatti, OH) at a 10:1 ratio with P3.X63.Ag8.653 (a BALB/c X 63 line which does not secrete immunoglobulin and which was a generous gift from Drs John Kappler and Philippa Marrack). The fused cells were placed into eight 96-well micro-culture plates (Costar 3596, Cambridge, MA), each well of which contained 4×10^3 CAF_1 peritoneal cells in 0·2 ml hypoxanthine, aminopterin and thymidine (HAT) containing medium. Cultures were grown for 2–3 weeks at 37° in 10% CO_2/90% air with fresh media changes twice weekly.

At 2–3 weeks post fusion, all wells were assayed individually for anti-Glo antibodies by ELISA (see below). Those wells (18/768) which contained anti-IgM Glo specificity showed no reactivity for IgM-Hoe. Two of these wells were cloned by limiting dilution into wells containing 10^5 CAF_1 spleen cells. Antibody activity was tested in those plated that demonstrated less than 15% growth. Clones JG-B1 and JG-B4, which originated from separate wells in the original plating, were transferred to media that lacked HAT and were grown as ascites in pristane-primed CAF_1 mice. Antibody was precipitated from ascites fluid by addition of Na_2SO_4 to a final concentration of 18%, the precipitate collected by centrifugation, solubilized in BBS and stored frozen.

Antisera

A panel of rabbit anti-mouse Ig reagents (Bionetics, Kensington, MD) was used to determine the isotypes of the cloned hybridoma antibodies. Sheep antiserum specific for mouse IgG (Cappel, Cochranville, PA) was radiolabelled with ^{125}I by a modification of the iodine monochloride method (Doran & Spar, 1980).

Assay

Hybridoma supernatants were screened by enzyme-linked immunoassay (ELISA) for anti-IgM Glo and anti-IgM Hoe antibodies. Fifty microlitres of the Ig to be tested (concentration 40 μg/ml) were coated onto polyvinyl 96-well plates (Costar 2595) by overnight incubation at 4° (Voller, Bidwell & Bartlett, 1979). Fifty microlitres of test supernatant were added and incubated for 2 hr. The presence of bound mouse antibody was detected by incubation with sheep $F(ab')_2$ anti-mouse Ig-β-galactosidase for 3 hr. (New England Nuclear, Boston, MA). After the addition of substrate, the yellow colour was assayed visually. Mouse Ig was included as a negative control since IgM-Glo has anti-IgG activity.

The clones were characterized using a more sensitive radioimmunoassay (RIA) performed in the same manner as the ELISA but replacing the enzyme-coupled reagent with ^{125}I labelled sheep anti-Mo-IgG antibody.

For one series of experiments, antibody precipitated from JG-B1 ascites was directly coupled to alkaline phosphatase (Sigma, St. Louis, MO) (Voller *et al.*, 1979) and used in a direct ELISA. The optical density of the wells were determined by using a Dynatech micro-ELISA reader.

RESULTS

Specificity of hybridomas

JG-B1 and JG-B4 were shown by immunodiffusion to be distinct antibodies with differing heavy chains, IgG2b-K, and IgG1-K respectively. Both JG-B1 and JG-B4 bound to Glo ($IgM\text{-}k_{IIIb}$) but not to Hoe ($IgM\text{-}K_{II}$) (Fig. 1). Therefore, the reagents appeared to be variable region specific. A more detailed characterization was done.

JG-B1 was tested on a panel of Igs to determine its binding specificity (Table 1). JG-B1 reacted with 5/8 of the mixed cryoglobulins tested. Glo, the immunogen, has been shown to belong to the V_KIII subgroup by sequence analysis (Johnston & Abraham, 1979) and the V_KIIIb sub-subgroup by heterologous antisera (Kunkel *et al.*, 1974). The IgG1-K myeloma All, and three other mixed cryoglobulins, all bound by JG-B1 have previously been assigned to the V_KIII variable

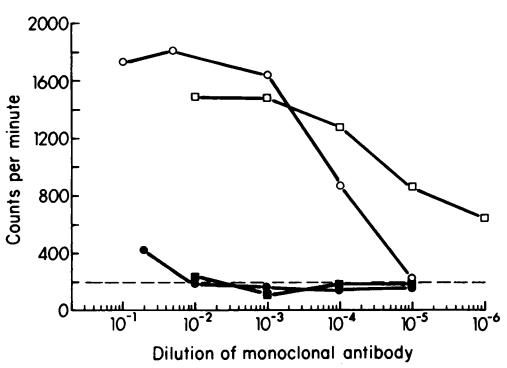

Figure 1. JG-B1 and JG-B4 bind to the immunogen (Glo) but not to the tolerogen (Hoe). JG-B1 (○) and JG-B4 (□) ascites were titrated in wells coated with either Glo (open symbols) or Hoe (closed symbols) followed by the addition of 125 I-sheep anti-mouse Ig. Data are representative of four experiments. (– – –), background binding of radiolabelled anti-mouse Ig to coated plates.

region subgroup by primary amino acid sequence analysis (Johnston & Abraham, 1979). Eight monoclonal human Igs (four Bence-Jones proteins, two IgG, and two IgA myeloma proteins) previously characterized serologically by one of the authors (A.S.) were tested as coded unknowns. Only those proteins (3/8) known to be V_KIIIb (Solomon & McLaughlin, 1969; McLaughlin & Solomon, 1972) were bound by JG-B1. One of the IgM anti-IgGs not bound by JG-B1 belonged to the V_KII subgroup (Abraham *et al.*, 1978). In summary, these experiments demonstrated that JG-B1 was specific for a determinant found only on V_KIIIb proteins.

When the same myeloma proteins were assayed, JG-B4 bound only to the immunogen, IgM-Glo (Table 1). JG-B4 also showed no reactivity with 20 other purified non-cryoglobulin rheumatoid factors purified by immunoadsorption (data not shown). These data suggested that JG-B4 was a non-cross-reactive anti-Glo idiotypic antibody.

Expression of JG-B1 and JG-B4 determinants on Ig fragments

The expression of the determinants defined by JG-B1 and JG-B4 on fragments of Ig molecules was examined by competitive solid-phase radioimmunoassay between whole Ig and Ig fragments. The ability of Fab, H and L chains isolated from IgG1-All to compete with intact IgG-All in binding JG-B1 is shown in Fig. 2. JG-B1 binding to All was inhibited by All Fab and L chain but not by H chain.

Glo L, H and half molecules were tested for the ability to inhibit JG-B4 binding to Glo, as shown in Fig. 3. JG-B4 binding was affected only by the addition of Glo half-molecules, suggesting that the idiotypic determinant was only expressed when the H and L chains of Glo were bound to each other.

Effect of antigen binding on the expression of JG-B1 and JG-B4 determinants

The preceding results demonstrated that the JG-B1 determinant was expressed on isolated L chains but that expression of the JG-B4 determinant required H and L chains to be associated. It was of interest to determine whether antigen binding by Glo would affect the expression of both JG-B1 and JG-B4 determinants. Glo (IgM anti-IgG) was coated onto polyvinyl wells and preincubated overnight with BSA, Glo antigen-IgG (purified from the cryoprecipitate) (Johnston & Abraham, 1979), or CR a heavy chain disease protein which behaves immunochemically as the Fc piece of an IgG1 (Franklin & Frangione, 1971b), and previously shown to be reactive with Glo. After this preincubation, JG-B1, JG-B4 and a control

Table 1. Specificities of JG-B1 and JG-B4 on a panel of immunoglobulin isotypes

		Bound by monoclonal antibody	
Immunoglobulin isotype tested		JG-B1	JG-B4
IgM mixed cryoglobulins	(IgM-k)	5/8	1/8
Myeloma proteins	IgM-k	0/2	0/2
	IgG_1-k	1/3	0/3
	IgG_1-λ	0/3	0/3
	IgG_2-k	0/4	0/4
	IgG_3-k	1/4	0/4
	IgG_4-k	0/1	0/1
	IgA-k	1/2	0/2
k Bence Jones proteins	k_{IV}	0/1	0/1
	$k_{III}a$	0/1	0/1
	$k_{III}b$	2/2	0/2

* Protocol as in Fig. 1, wells were coated with each protein tested.
† The serologic properties of the proteins assayed are provided in the Appendix.

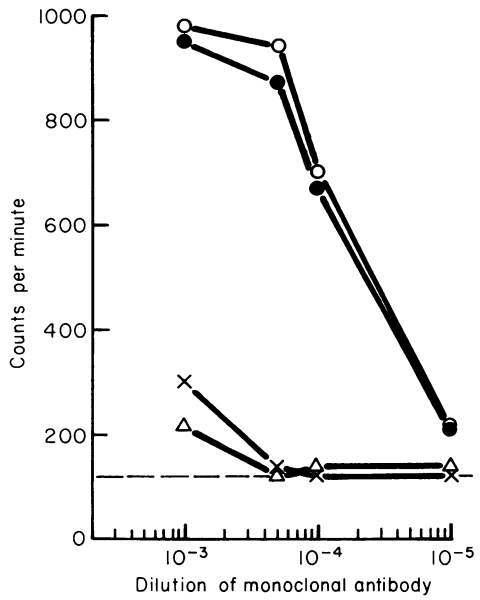

Figure 2. JG-B1 binding is inhibited by free L chain and Fab but not Fc. JG-B1 binding to ALL was examined in the presence of 30 μg/well: BSA (●), All Fc (○), All Fab (×), or All L chain (△). The experiment shown is representative of two.

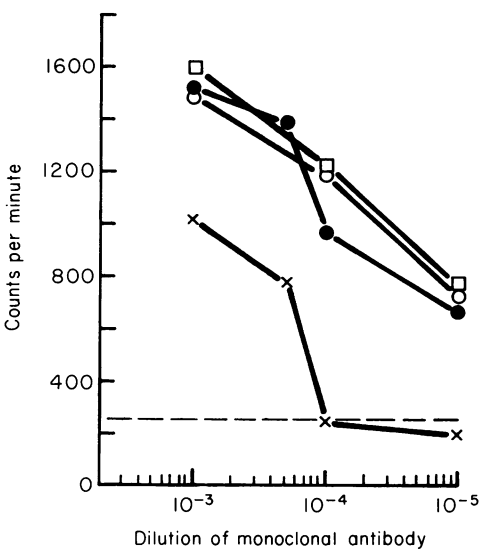

Figure 3. JG-B4 binding is inhibited by mildly reduced and alkylated Glo but not free Glo H or L chains. JG-B4 binding to Glo as examined in the presence of: 40 μg/well reduced and alkylated Glo (×), 40 μg/well BSA (●), 25 μg/well Glo L chain (○), or 50 μg/well Glo H chain (□).

monoclonal anti-K antibody were assayed for reactivity with IgM-Glo (Fig. 4A, B). JG-B1 and JG-B4 binding was unaffected by addition of BSA (Fig. 4A, B). However, incubation with CR and Glo antigen-IgG blocked the ability of JG-B1 to bind Glo. In control experiments it was shown that neither JG-B1 or JG-B4 reacted with CR or Glo antigen-IgG alone (data not shown). Similarly, binding of JG-B4 (Fig. 4B) was blocked by Glo binding either CR or Glo antigen-IgG.

JG-B1 and JG-B4 binding to Glo was more completely blocked by Glo antigen-IgG than by CR. As expected, reactivity of the anti-K antibody with IgM-Glo was completely blocked by Glo antigen, but not CR. Since Glo anti-IgG is much larger than CR, the difference in blocking ability may be due to steric hindrance or selective reactivity of IgM-Glo with gamma-2 heavy chains.

Cross-blocking of JG-B1 and JG-B4

Since Glo expresses both the JG-B1 and JG-B4 determinants, it was possible to assay whether Glo would simultaneously react with both JG-B1 and JG-B4. Microtitre wells coated with IgM-Glo were incubated with a 10^{-4} dilution of JG-B1 and various dilutions of JG-B4 (Fig. 5). At high concentrations of JG-B4, the amount of monoclonal antibody (JG-B1 and JG-B4) bound to Glo was less than the sum of

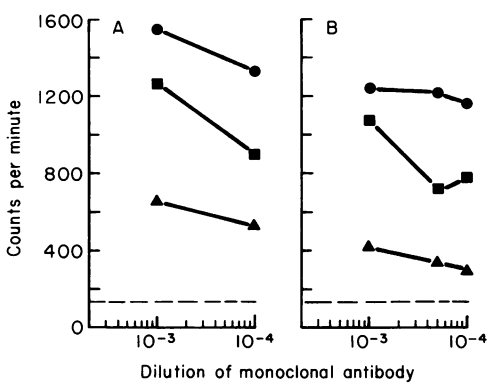

Figure 4. The binding of JG-B1 and JG-B4 is blocked when Glo is bound to its antigen. Wells were coated with Glo followed by overnight incubation with 50 μg/well: BSA (●), IgG purified from Glo cryoglobulin (▲) or Cr (■) (a heavy chain disease protein which has been shown to be bound by Glo). Dilutions of JG-B1 (panel A) or JG-B4 (panel B) were then added, followed by ^{125}I-sheep anti-mouse Ig. The experiment shown was representative of three.

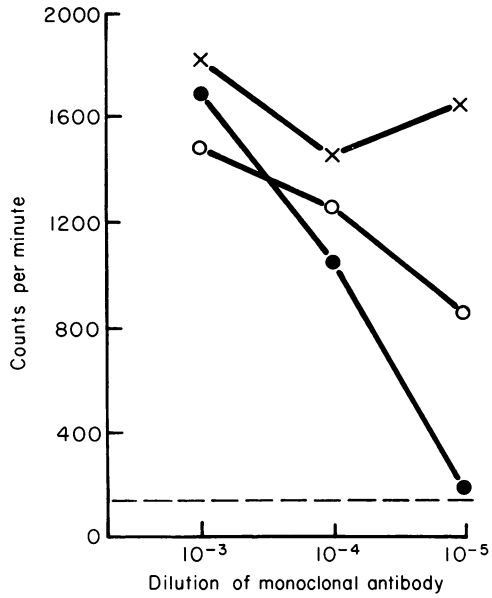

Figure 5. JG-B1 binding competitively blocks the binding of JG-B4 to Glo. JG-B1 (○), JG-B4 (●), or JG-B4 + 10^{-4} dilution of JG-B1 (×) were titrated in wells coated with Glo. Data shown are representative of three experiments.

JG-B1 and JG-B4 binding when these were added alone. With lower concentrations of JG-B4, the amount of JG-B1 and JG-B4 bound was approximately equal to the sum of each alone. These results suggest that at saturating concentrations of JG-B4, the ability of JG-B1 to bind IgM-Glo was blocked.

In order to examine this question more directly, JG-B1 was coupled to AP (AP-JG-B1) and the ability of JG-B4 to block the binding of AP-JG-B1 to Glo was tested (Fig. 6A). JG-B4 incompletely blocked the ability of AP-JG-B1 to bind Glo, whereas JG-B1 blocked all the AP-JG-B1 binding sites. This finding suggested that IgM-Glo possessed two available JG-B1 sites for each JG-B4 site.

DISCUSSION

Monoclonal antibodies which are highly specific for a single human variable (V) region subgroup determinant have not been previously described. These are among the first experiments to characterize their reactivity. Recently, Kubagawa *et al.* (1982) attempted to produce monoclonal antibodies specific for human V_H region subgroups by immunization of mice with $V\mu$ fragments, μ chains or whole myeloma proteins. These monoclonal antibodies reacted with unique V_H region determinants, but the antigens detected were common to more than one V_H region subgroup and were found only on free H chains and not intact immunoglobulin.

In the present study a modified immunization protocol was used. In order to generate variable-region specific monoclonal antibodies, mice were

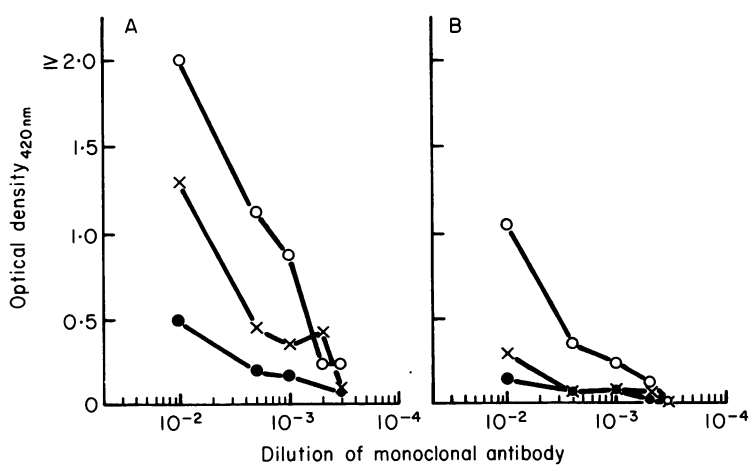

Figure 6. JG-B4 binding to Glo did not completely block the ability of JG-B1 to bind Glo. Wells coated with either Glo (panel A) or reduced and alkylated Glo (panel B), and were incubated with various dilutions of JG-B1 (●), JG-B4 (×), or control buffer alone (○). This was followed by the addition of various dilutions of AP-coupled JG-B1. The data are representative of three experiments and show that JG-B4 blocks one-half and JG-B1 all of the AP-JG-B1 binding sites. This indicates that intact Glo possesses two available JG-B1 sites for each available JG-B4 site.

injected with a tolerizing dose of a human IgM-K prior to their immunization with the Glo-IgM anti-IgG. This simple procedure appeared to decrease the number of murine anti-μ and anti-K producing hybridomas and enriched the response for monoclonal anti-variable region antibodies. When this was not done, fusions yielded only anti-μ and anti-K antibodies (data not shown).

The hybridomas JG-B1 (anti-V_KIIIb) and JG-B4 (anti-idiotype) each demonstrated a distinct and highly specific reactivity pattern as shown by analysis with a panel of mixed cryoglobulins and myeloma proteins. JG-B1 could discriminate proteins of the V_KIIIa and V_KIIIb sub-subgroups which were previously defined by use of heterologous antisera (Solomon & McLaughlin, 1969; McLaughlin & Solomon, 1972). Concordance in identification of V_KIIIb proteins with heterologous anti-V_KIIIb antisera was substantiated since JG-B1 reacted with the Xoc Bence-Jones and IgA-Xoc, proteins.

In contrast to the specificity of JG-B1, JG-B4 was very different in reactivity as compared to polyclonal antisera with similar gross specificities. JG-B4 only reacted with the IgM-Glo idiotype as expressed on intact Glo and its half-molecules. It identified an idiotypic determinant formed by the association of Glo heavy and light chains which was not present on any other anti-IgG or myeloma protein tested. IgM-Glo has been shown by Kunkel *et al.* (1973) to belong to the major Wa anti-IgG cross-reactive idiotype group and to contain V_KIIIb light chains (Kunkel *et al.*, 1974). Both characteristics were determined with highly absorbed heterologous rabbit antisera. No experiments were described which tested the influence of antigen binding on expression of both the V_KIIIb and idiotypic determinants. Because of the monoclonality of JG-B1 and JG-B4 and their precise and restricted specificity patterns, these experiments were able to be performed.

Despite differences in their fine specificity, JG-B1 and JG-B4 were both competitively inhibited by antigen binding. When IgM-Glo reacted with either its antigen-IgG or the heavy chain disease protein CR, the binding of both JG-B1 and JG-B4 was inhibited. However, Glo antigen-IgG completely inhibited while CR only partially inhibited, binding of the monoclonal antibodies. This difference is felt to be due either to size differences between the antigen-IgG and CR, or the preferential reactivity of IgM-Glo with γ2 (antigen-IgG) as opposed to γ1 (CR) heavy chains (Johnston & Abraham, 1979).

A similar finding was reported by Kunkel *et al.* (1976) for heterologous anti-idiotypic 'like' and anti-variable region antisera. Fab fragments prepared from IgG anti-Rh antibodies, showed reactivity with both cross-reactive anti-idiotypic antisera and antisera specific for some heavy and light chain variable region subgroups. However, the reactivity with both types of antisera was blocked when the Fab anti-Rh antibodies were reacted with Rh (+) human erythrocytes. Of significance in this regard were control experiments (data summarized above) which showed that intact IgG blocked reactivity of a monoclonal anti-K reagent with IgM-Glo while CR did not. These findings show that antigen blocking of the reactivity between anti-idiotype:idiotype may not always indicate that an anti-idiotypic reagent is hypervariable region or antibody combining-site specific.

The relationships between the binding of each antibody with its antigenic determinant were also clarified by use of a quantitative competitive inhibition assay. The data show that JG-B1 and JG-B4 competitively inhibit the binding of each other to IgM-Glo and suggest that the antigenic determinants recognized are most likely close to each other, i.e. within the same region of the Fab. Anti-idiotype reagents have been previously shown to cross-inhibit anti-V_KIIIb binding (Kunkel *et al.*, 1976).

When tested directly, the JG-B1 and JG-B4 cross-inhibition was only partial with intact Glo, but when cleaved into half-molecules equal numbers of idiotypic and V_KIIIb determinants are expressed.

The reagents described here and others currently being characterized with similar types and restrictions in their specificities will allow the further characterization of variable region subgroup expression and an understanding of the variable region restriction in autoantibodies (Ledford *et al.*, 1982). Perhaps the B cells expressing V_KIIIb Ig have a mechanism to escape the inherent anti-self controls. With a panel of monoclonal anti-variable region reagents, it is our hope to understand the control of the restriction of the human autoimmune response.

APPENDIX

The following IgM anti-IgG cryoglobulins or myeloma proteins were assayed. In these studies variable region subgroups have been previously determined by either primary amino acid sequence analysis or reactivity with antisera specific for the V_KIIIb determinant (nd = not yet determined).

IgM mixed cryoglobulins:
IgM V_KIII: Glo, Arn, Cra, The, Pla
V_KII: Glz
V_Knd: Sau, Zwi

Myeloma proteins:
IgM: V_KI = Har; V_KII = Hoe
IgG1K: V_KI = Ric, Woo; V_KIII = All
IgG1λ: VλIII = Mit, Lon; Vλnd = Pie
IgG2K: V_KI = Web, Pig; V_Knd = Thib, Per
IgG3: V_KI = Cant, Coo; V_KIIIb = Will
IgG4K: V_Knd = Dun
IgA-K: V_KIIIb = Xoc; V_Knd = Wash

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