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Y Ben-Neriah; ... et. al

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PREPARATION AND CHARACTERIZATION OF ANTI-FRAMEWORK ANTIBODIES TO THE κ-CHAIN VARIABLE REGION (V_κ) OF MOUSE IMMUNOGLOBULINS[1]

YINON BEN-NERIAH, PETER LONAI, AND DAVID GIVOL[2]

From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

The variable fragment of mouse kappa light chain (V_κ) was prepared from myeloma protein XRPC-25. The preparation included tryptic digestion of the light chain, anion exchange chromatography, and preparative SDS polyacrylamide gel electrophoresis. The end product was identified by N terminal amino acid sequence and was proven to be pure V_κ by both sequence analysis and size.

Rabbit antiserum raised against the purified V_κ fragment, precipitated the V_κ25 fragment, the L25 chain, and only slightly the intact myeloma protein X-25. In double diffusion in agar against anti-V_κ, a line of identity was formed between L25 and V_κ25. In radioimmunoassay, complete inhibition of L25 binding by V_κ25 was observed. This fact and the lack of cross-reaction with V_λ 315 or with heavy chains demonstrate the V_κ specificity of the antiserum. Other κ-chains did not give precipitin line in double diffusion but were found to cross-react with anti-V_κ in radioimmunoassay, regardless of their subgroup or their respective antibody specificity. Pooled light chains from either BALB/c or AKR Ig also shared anti-V_κ25 specificities. Anti-V_κ25 was absorbed completely by Sepharose-pooled L chain. It follows that the antibody is specific to common V_κ framework-related determinants and denoted variotypes, and may be useful for the detection of V_κ domains in products or receptors of lymphatic cells. This anti-V_κ antibody complements a series of V-region-specific antibodies, the first two of which, anti V_H and anti V_λ, were already found to be useful in probing the presence of V regions on T cells.

Only a few V-region markers are known for detection and characterization of mouse Ig and lymphocyte receptors. Most markers are V_H[3]-associated idiotypes and are suitable for limited research systems dealing with certain unique antigenic specificites such as phosphorylcholine in BALB/c mice (1), a carbohydrate in A/J mice (2), *p*-azophenylarsonate in A/J mice (3), and a few others (4). V-region markers have not been known for mouse λ-chains, and very few are known for κ-chains such as the Ib peptide (5) or antigenic determinants of the $V_{\kappa 21}$ subgroup (6). All these markers are present in a very small portion of the Ig repertoire, and their use for detecting V regions on T cells must be limited to either functional tests or to a small percentage of the cells. We have recently introduced anti-V-region antibodies that can detect common antigenic determinants on different heavy (V_H) or lambda (V_λ) chain V regions. These antisera were shown to detect framework-related V-region antigenic determinants (7, 8) and are being used now to detect V regions on T cells and in particular to inhibit several of their functions such as helper and suppressor functions.

The anti-V-region antisera were prepared by xenogeneic immunization with purified V_H or V_λ (7, 8). To complement these antisera, we now report the preparation of rabbit anti-mouse V_κ antibodies. A prerequisite for the preparation of anti-V-region framework antibodies is the isolation of a purified V fragment devoid of any C region or intact light chain contaminants. Xenogeneic antisera against such purified fragments need not be adsorbed with unrelated Ig and thereby include cross-reactive antibodies to many V regions.

This report describes the preparation of V_κ25 from the myeloma protein XRPC 25 and the characterization of rabbit antibodies raised against this fragment. Anti V_κ25 was found to be specific for V_κ antigenic determinants and to cross-react with many L chains. Thus, we now have a set of anti-V-region reagents specific for either mouse V_H, V_λ, or V_κ domains that are suitable for screening different V domains associated with lymphatic cell products and receptors. Initial efforts in the use of these reagents in analyzing T cell receptors have been recently reported (9–11).

MATERIALS AND METHODS

Myeloma proteins and mouse IgG. Proteins MOPC-315 (α, λ_2, M315), XRPC-25 (α, κ, X25) and MOPC-460 (α, κ, M460) were purified from ascites fluids of the respective plasmacytomas on 2,4-dinitrophenyl (DNP)-lysine-Sepharose. Protein MOPC-104E (μ, λ_1, M104E) was purified from a 45% saturated ammonium sulfate precipitate of ascites fluid on Ultrogel ACA-22 (LKB Bromma, Sweden). Protein MOPC-167 (M167) was a gift of Dr. M. Potter. Protein UPC-10 (U10) was a gift of Dr. I. Pecht, and the light (L) chain of MOPC-321 (L321) was a gift of Dr. I. Schechter. We acknowledge the continuous help of Dr. M. Potter (NIH) in supplying tumor lines.

Mouse IgG (MIgG) was obtained by precipitating mouse

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[2] Send correspondence to: David Givol, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.
[3] Abbreviations used in this paper: L, light chain; H, heavy chain; Fv, variable fragment of Ig; V_L, variable fragment of L; V_H, variable fragment of heavy chain; L25, L10, L167, L321, light chain of the respective myeloma proteins; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DNP, 2,4 dinitrophenyl; PTH, phenylthionydantoin.

serum at a 45% saturation of ammonium sulfate followed by chromatography on DEAE-cellulose in 0.02 M sodium phosphate, pH 7.6. Only the unadsorbed protein was collected; analysis by electrophoresis on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) demonstrated that only H and L chains were present.

Preparation of H and L chains. Preparation of H and L chains of Ig were performed as described (7). Separated chains were dialyzed against a 2 x 30-fold volume of water, filtered through a Millipore filter (0.45 μm), and stored at 4°C. Before use, H or L chains were diluted in phosphate-buffered saline (PBS) to the desired concentration. Under these conditions, H or L chains (0.5 mg/ml) remained soluble for weeks. Preparation of V_λ from Fv 315 was performed as described previously (12). The purity of V_λ was checked by SDS-PAGE as described (13).

Preparation of V_κ. $V_\kappa 25$ was prepared from L25 as follows: L chain of XRPC-25 myeloma protein (10 mg/ml in 0.1 M Tris-Cl, pH 8.2) was digested by trypsin (1:50 w/w) for 45 min at 37°C; and the reaction was terminated by the addition of soybean trypsin inhibitor (1:1 in relation to trypsin). The digest was dialyzed against 0.05 M NH_4HCO_3 and chromatographed on DEAE-cellulose equilibrated with this buffer. Most of the absorbance at 280 nm was eluted with 0.12 M NH_4HCO_3 and was found to contain mainly a fragment of 13,000 m.w. and some undigested L chain. Further electrophoresis on a preparative SDS-PAGE (Savant) separated the 12,000-m.w. fragment from L chain. The purity of the fragment was tested by PAGE and N-terminal sequence.

Anti-$V_L 25$. Rabbits were injected three times with 0.25 mg $V_L 25$ in complete Freund's adjuvant with 2 weeks interval between injection, and the animals were bled 10 days after the last injection. Antibodies were purified by affinity chromatography on L25-Sepharose and elution with 0.2 M acetic acid. The antibody content was between 0.3 and 0.5 mg/ml.

Radioimmunoassay. Double antibody radioimmunoassay was performed in duplicates with [^{125}I]-labeled L25 chains. L25 was iodinated by the chloramine-T method as described (14). Specific activity of the labeled chains was 4×10^6 cpm/μg. Anti-V_κ antibodies (0.1 to 10 μg, supplemented with normal rabbit Ig to 10 μg) were incubated with ^{125}I L chain (20,000 cpm) for 10 min at 37°C, followed by the addition of 40 μl goat anti-rabbit Ig. After further incubation for 10 min at 37°C and 4 hr at 4°C, the reaction tubes were centrifuged, washed twice with cold BSA-PBS, and the precipitates were counted in a Packard autogamma spectrometer. Eighty-five per cent of the radioactivity was precipitated by the antibodies, and the point of 60% antigen precipitation (corresponding to 2 μg anti-V_L antibody) was selected for inhibition studies. Radioimmunoassay inhibition was performed as above except that the inhibitor was added to the anti-V_κ 10 min before the labeled [^{125}I] L-chain.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed in slab gels containing 15% polyacrylamide and 0.1% SDS as described by Laemmli (15). Preparative electrophoresis in a cylindrical gel (15%) was performed in an apparatus purchased from Savant Instrument Inc., according to the manufacturer's directions. Buffers were the same as in slab gels. The sample of L25 tryptic digest was mixed with a small amount of [^{125}I]-labeled digest (10^6 cpm), and 2-ml fractions from the gel electrophoresis were collected in an LKB fraction collector. The fractions were assayed for their radioactivity, OD_{280}, and also by slab SDS-PAGE.

Amino acid N-terminal sequence. This was performed on Beckman Model 890 C Automatic Sequencer by a modification (16) of the method of Edman and Begg (17). The phenylthiohydantoin (PTH) amino acids were identified quantitatively by gas liquid chromatography (18).

RESULTS

Preparation and characterization of $V_L 25$. The tryptic digest of L25 contains mainly undigested L and a fragment of approximately 13,000 m.w. that previously was identified as V_L since it can recombine with H25 to yield a molecule with DNP-binding specificity (19). Attempts to isolate the 13,000 m.w. fragment from L25 on Sephadex G-75 or G-50 in acetic acid or in 8 M urea were unsuccessful. A previously described method (7) to prepare $V_\kappa 25$ by reassociation with H25 followed by separation on G-75 in 4 M urea in 1 M acetic acid gave a poor yield. Hence we prepared $V_\kappa 25$ by electrophoresis of 5 mg of the tryptic digest on a cylindrical polyacrylamide gel in 0.1% SDS (Fig. 1). Routinely, a small amount of [^{125}I]-labeled digest (10^6 cpm) was added to the sample, and the fractions (2 ml) were assayed for their radioactivity to detect the positions of various fractions (Fig. 2). Fractions were pooled, dialyzed against water, lyophilized, and analyzed on a slab PAGE (Fig. 2).

The 13,000-m.w. fragment was further analyzed for its N-terminal sequence by 10 cycles in the sequencer, and the results showed the following sequence:

Asp-Ile-Val-Leu-Thr-Gln-Ser-Pro-Ala

which is identical to the N-terminal sequence of L25 (20). The repetitive yield was 92%, and there was no indication of any

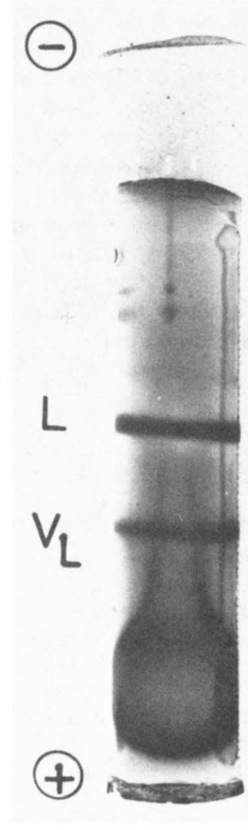

Figure 1. Preparative SDS-PAGE of a tryptic digest of L25. A sample of 1 mg digest was electrophoresed at 150 volts. After 2 hr of electrophoresis, the gel was stained and photographed. Digest was supplemented with 1 mg of undigested L25 in order to facilitate the detection of L chain. Gel was 15% in polyacrylamide and 0.1% in SDS.

contaminants in the PTH amino acids at each step. Hence the 13,000-m.w. fragment must be the V_κ domain of L25.

V-region specificity of anti V_κ25. Figure 3 shows that V_κ25 and L25 formed a line of identity in double diffusion against anti V_κ25. Thus V_κ25 is not antigenically deficient to L25 and the C_L determinants of L25 are probably not recognized by the antiserum. Since the V_κ fragment was proven to be pure both electrophoretically and by sequence analysis, it is unlikely that the antibody contained anti-C_κ activity.

Anti-V_κ25 shows poor precipitation with the intact myeloma protein X-25 in a double diffusion assay in agar (Fig. 3). Aside from L25, no other L chain was precipitated by the antiserum. This is in contrast to the regular precipitating activity of anti C_κ sera with different κ-chains, indicating that the antiserum does not recognize C_κ determinants. The inhibition of the reaction between anti-V_κ25 and [^{125}I] L25 was analyzed in radioimmunoassay (Fig. 4). It is shown that V_κ25 completely inhibits the reaction indicating that anti-V_κ25 recognizes only V_κ determinants. On a molar basis, V_κ25 inhibits 10 times better than L25, suggesting that either some of the determinants on V_κ25 may have different conformations in V_κ25 and in L25, or they are more accessible due to the absence of the C region.

V_κ specificity of the antiserum. Anti-V_κ25 did not precipitate V_λ315 in a double diffusion assay (Fig. 3), and V_λ315 or L315 did not compete with [^{125}I] L25 for anti-V_κ25 in radioimmunoassay. Adsorption of anti-V_κ25 on V_λ315-Sepharose did not alter its reactivity to V_κ25 (Fig. 4). Anti-V_κ25 also did not react with H chains (Fig. 4). No inhibition was found with H315 (α), and the slight inhibition by H25 (which is 100 fold less than L25) is probably due to a small contamination of H25 by L25. On the other hand, four different mouse L_κ chains inhibited the reaction between anti-V_κ and [^{125}I] L25 (Figs. 4 and 5).

The various L chains (L460, L10, L167, and L321) belong to different V_κ subgroups, and they inhibited the homologous reaction to an extent of 40 to 95% in our assay. It is suggested therefore that anti-V_κ25 is mainly directed to framework-related determinants. This general V-region specificity, which is different from anti-idiotypic specificity, is further illustrated by the complete inhibition of anti-V_κ25 by L chains derived from normal mouse sera, either of BALB/c or of AKR/J (Fig. 5).

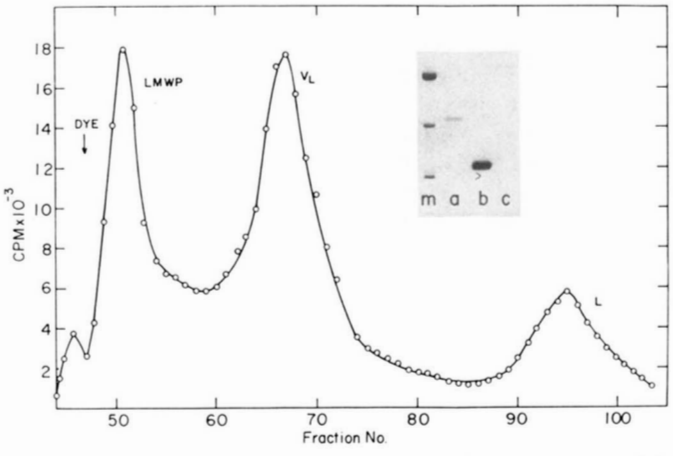

Figure 2. Preparation of V_κ25 by PAGE. L25 digest was partially purified by DEAE cellulose chromatography and 5 mg were subjected to a preparative SDS-PAGE (9 x 1.5 cm). The radioactive fractions were collected and identified on a slab gel (*inset*). LMWP, low molecular weight peptides; *m*, markers from top to bottom: ovalbumin (m.w. 43,000), chymotrypsinogen (m.w. 23,500), lysozyme (m.w. 14,500). *a*, Light chain; *b*, V_κ; *c*, LMWP.

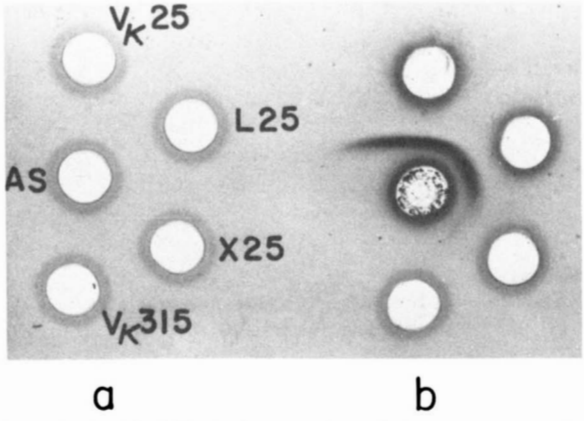

Figure 3. Double diffusion in agar of anti-V_κ25 antiserum against the indicated antigens. Center well contained anti-V_κ25 (*b*) or anti-V_κ25 previously adsorbed on pooled mouse L chain-Sepharose (*a*). Antigens' concentrations were V_κ25, L25, V_λ315, 50 μg/ml; X25, 0.5 mg/ml, and the antigens in *b* are the same as in *a*.

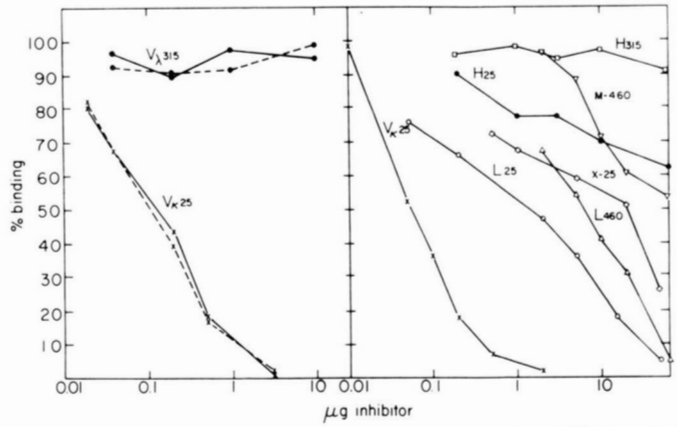

Figure 4. Radioimmunoassay of anti-V_κ25 antibodies. [^{125}I]-labeled L25 (20,000 cpm) were incubated with 2 μg of anti-V_κ antibodies followed by goat anti-rabbit Ig. Sixty per cent of the cpm were precipitated when no inhibitor was added and nonspecific precipitation by normal rabbit Ig was less than 3%. For details of inhibition see *Materials and Methods* section. *Left, full line*, unadsorbed anti-V_κ; *dashed line*, the used of anti-V_κ25 antibodies, previously adsorbed on V_λ315-Sepharose. *Right*, inhibition of the binding of the V_κ25 by anti V_κ by various immunoglobulins or chains.

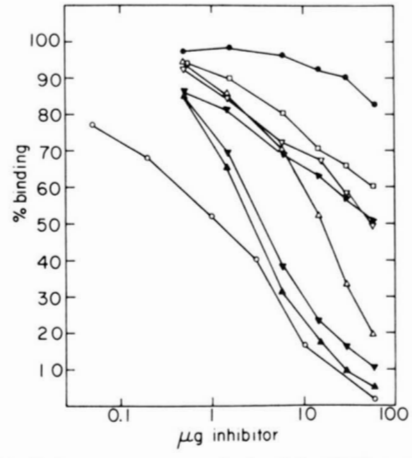

Figure 5. Radioimmunoassay analysis of the V_κ25 cross-reactive variotypes among mouse light chains. For details see legend to Fig. 4 and *Materials and Methods* section. Inhibitors used were: ○, L25; ▲, L-BALB/c (pooled serum L chains); ▼, L-AKR/J; △, L460; ▶, L321; ▽, L167; □, L10; ●, L315.

Anti-idiotypic antibodies are regularly adsorbed on normal mouse Ig to render them idiotype specific, but they nevertheless retain the anti-idiotypic titer. On the other hand, anti-V_κ25 adsorbed on normal mouse L chains neither precipitated L25 in a double diffusion assay (Fig. 3) nor did it bind [^{125}I] L25 in a radioimmunoassay (results not shown). This indicates that anti-V_κ25 is directed against common V_κ determinants shared by many L chains.

DISCUSSION

In recent publications, we have reported the preparation and characterization of antisera raised against the V-region fragments, V_H and V_λ, of the myeloma protein M315 (7, 8). Such sera were found to be directed to framework-related determinants of mouse H or λ-chain variable regions, respectively, and could be used to screen these V domains in various experimental systems (9–11). This report describes the preparation and characterization of another antiserum raised against the V_κ fragment of myeloma protein X25, (α, κ), which is shown to be an anti-framework antibody, useful for detection of V_κ domains on immunoglobulins.

The V region specificity of the antiserum is evident from the complete identity of the precipitin lines formed between V_κ25 and L25 against anti-V_κ25 (Fig. 3). On the other hand, the antibodies do not precipitate heterologous κ-chains indicating that they are not directed against the constant region. Several other results exclude anti-C_κ activity in the antibody preparation: a) in the radioimmunoassay V_κ25 inhibits completely (in fact 10- to 15-fold better than L25) the reaction between [^{125}I] L25 and anti-V_κ (Fig. 4). b) L chains other than L25 competed between 5 and 60-fold less effectively than L25 for anti-V_κ25 antibodies. If the cross-reactivity was due to minor anti-C_κ activity, there would be no reason for the differences in inhibition capacity (Fig. 5). The reason for lack of precipitation in double diffusion between anti-V_κ and heterologous κ-chains is not completely clear, although such cases are known in several systems of cross-reactive antigens (e.g. see References 8 and 35). Immunodiffusion will reveal only insoluble complexes between antigens and high affinity antibodies, whereas radioimmunoassay or immunoadsorption reveal both high and low affinity antibodies.

As in the case of the other anti-V-framework sera described (7, 8), the V-region specificity of the antiserum was directed against the products of one mouse V gene pool, V_κ in this case. This is evident from the lack of inhibition by λ-chain of the reaction between anti-V_κ and L25 (Fig. 4). An attempt to adsorb anti-V_κ activity on V_λ315-Sepharose did not result in any change in the activity of the antibodies (Fig. 4). Similarly, H315 chains could not inhibit the binding of L25 to anti-V_κ25 antibodies (Fig. 4).

Anti-V_κ25 displays extensive cross-reactivity with various L chains (Fig. 5), which must be due to the V_κ-region determinants. The mouse V_κ is an extremely heterogeneous group (21) composed of as many as 50 subgroups (isotypes) (22, 23). We have examined several L chains that belong to various distantly related V_κ prototypes (21) for common V-region determinants, by their cross-reactivity with V_κ25. All κ-chains tested could effectively inhibit anti-V_κ25 antibodies (Fig. 5). L460, which is different from L25 by 22 out of the 33 N-terminal residues, or evolutionary distant from L25 by a minimum 22 genetic events (24), could inhibit completely the binding of [^{125}I] L25 to anti-V_κ25. Interestingly, the other L chains tested are not more distant evolutionary from L25 than L460. L10 differs from L25 by only 14 positions out of the 33 N-terminal residues and is 18 genetic events distant from L25 (24). Yet L10 is approximately 6-fold less efficient than L460 in competing with L25 for anti-V_κ25 antibodies. Hence, L460, an anti-DNP-derived L chain, can be distinguished from other non-anti-DNP-derived κ-chains in its antigenic relationship to the anti-DNP-derived L25. This may suggest some structural relationship between anti-DNP L chains in analogy to the framework sequence relationship of anti-DNP H chains (24). It is possible that the sequence of the entire V_κ region may indicate such a relationship, which will modify the relatedness tree (24) based only on the first 34 residues.

The nearly complete inhibition of anti-V_κ25 by L460 excludes anti-idiotypic activity of the Idi type (4) from anti-V_κ25. This is similar to the case of the anti-V_H antibodies raised against the isolated V_H315 fragment (8) and distinct from other cases reported in the literature such as the anti-Idx described by Bosma *et al.* (25). In the latter case, an antiserum made against Fab of M173 cross-reacted with the unrelated myeloma protein U10 but not with many other myeloma proteins even if they were of the same subgroup.

Several other anti-V region antisera showing cross-reactivity with different V regions are known. Antisera specific to human V_H subgroups were prepared by injecting myeloma proteins or their $F(ab')_2$ into rabbits followed by absorption with myeloma proteins of other subgroups (26). These antisera were found to be specific to V-region subgroups. Antisera against human V_L region were prepared by Solomon and McLaughlin (27), and serologic typing of human V subgroups was performed by these authors (28) and by Tischendorf *et al.* (29).

Unlike the above mentioned cases, the anti-V_κ25 described here was prepared against the isolated V_κ domain, and the antiserum was not adsorbed on any Ig. We think that the use of an isolated V domain is a prerequisite for the generation of anti-V region with broad specificity because a) absorption with Ig in order to remove anti-C region antibodies is not necessary and therefore the antibodies that cross-react with other V regions are not being removed; and b) it is also possible that some V-region antigenic determinants may be more immunogenic on the isolated V region than on L chains or Fab, because of antigenic competition or for structural reasons (exposure, e.g.).

Anti-idiotypes usually react only with the unique V_H - V_L pair, which was used as the immunogen, and in most cases, reassociation with heterologous H or L chains will abolish the idiotypic determinants (30–34). In our case, the chance of cross-reactivity with many V regions is larger because the determinants are not dependent on a particular V_H - V_L association. The fact that anti-V_κ25 is completely absorbed by pooled L chain-Sepharose, indicates that the antibodies are highly cross-reactive with many V regions of L_κ. Indeed, our antibodies cross-react with L chains of different subgroups and prototypes derived from myeloma Ig of different specificities (DNP, levan, phosphorylcholine). Therefore they may be defined as anti-framework-related determinants and can be used as probes for V_κ regions.

Anti-V-region framework antibodies have been recently used to probe receptors of T lymphocytes (9–11). Several interesting features of the structure and regulation of T cell receptors became evident from these studies: a) The antigen recognition structures of T cells contain the V-region characteristic framework in addition to the previously reported idiotypes (34). b) V-region domains of H or L chains are selectively expressed in receptors of T cells, in contrast to the mutual expression of V_H and V_L domains in B cells (9, 11). c) Soluble receptor material

released from T cells (helpers or suppressors) is also associated with V-region framework determinants.[4] (10). The availability of anti-V_κ framework antibodies provides an additional tool to study the V region of L_κ on T lymphocytes.

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