Immunology 1985 **54** 207

The V_KIIIb light chain sub-subgroup: restricted association with mu heavy chain in normal human serum

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Accepted for publication 7 September 1984

Summary. The V_KIII human kappa light chain subgroup has been serologically and structurally divided into two sub-subgroups, V_KIIIa and V_KIIIb. V_KIIIb has been shown by others to be strikingly prevalent in IgM autoantibodies, but no studies have been performed to determine heavy chain isotype association with V_KIIIb light chain in normal human serum. The V_KIIIb sub-subgroup was shown here to be associated with mu heavy chain in normal human serum, but was not detected in association with gamma or alpha heavy chain. Approximately $25 \pm 15\%$ of IgM-kappa was determined to be V_KIIIb. Both intact IgG and purified light chains from pooled IgG did not bind monoclonal anti-V_KIIIb, indicating that the determinants recognized by anti-V_KIIIb are not merely masked in intact IgG. These results are the first report of a light chain sub-subgroup showing preferential association with a heavy chain isotype.

INTRODUCTION

In humans, the V_KIII light chain subgroup has been structurally and serologically classified into two sub-subgroups, designated V_KIIIa and V_KIIIb (Solomon & McLaughlin, 1971). Studies of V_KIIIb expression have generally focused on their percent distribution in a randomly selected population of immunoglobulin paraproteins (Kunkel *et al.*, 1974) and their presence in particular monoclonal IgM autoantibodies found in some human autoimmune disease states. In the latter category is a unique and predominant association of V_KIIIb light chains with IgM cold agglutinins (Capra *et al.*, 1972; Feizi *et al.*, 1976) and IgM anti-IgG cryoglobulins (Kunkel *et al.*, 1974; Ledford *et al.*, 1983). There are no studies which quantify the amount of V_KIIIb in normal human serum and determine its expression in each of the major immunoglobulin classes. Such information might be an important first step in understanding the prevalence of V_KIIIb in autoantibodies. By use of a previously characterized murine monoclonal anti-V_KIIIb antibody (Greenstein, Solomon & Abraham, 1984), the percent of V_KIIIb light chains in K light chains was determined for IgG, IgM and IgA in sera of six normal volunteers.

Abbreviations: BBS, borate-buffered saline: BSA, bovine serum albumin; CF II, Cohn fraction II; DEAE, diethylaminoethyl; ELISA, enzyme-linked immunoassay; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Proteins

ALL (IgG1-V_KIIIb) and MIT (IgG1-λ) myeloma proteins were precipitated from plasma in 20% Na_2SO_4 and, after dialysis *vs* 0·015 M phosphate buffer, pH 7·4, were purified as the fall-through peak after chromatography on a DEAE cellulose column equilibrated with the same buffer. Cohn fraction II (CF II) was purchased from Sigma Chemical Co. (St

Louis, MO). Normal human IgM and IgG were obtained as previously described (Johnston, Abraham & Welch, 1975). After initial chromatography of serum on Sephadex G-200, the IgG-containing peak, as determined by immunodiffusion in agar gel, was dialysed against 0·015 M phosphate buffer and purified IgG obtained as the fall-through peak after DEAE chromatography. Light chains were isolated by chromatography of reduced and alkylated pooled IgG on a Sephadex G-100 column equilibrated with 1 M acetic acid (Johnston & Abraham, 1979). A V_KIIIb Bence-Jones protein was isolated from the urine of patient LAR as follows. Urine was extensively dialysed *vs* 0·015 M phosphate buffer, pH 7·4, the dialysate placed on a DEAE column equilibrated with the same buffer, and the LAR light chains eluted in the fall-through. The LAR light chains were reduced and alkylated as described. All proteins were shown to be immunologically pure by immunodiffusion and immunoelectrophoresis utilizing equine polyvalent anti-whole human serum antiserum and rabbit antisera specific for the gamma G, A, and M heavy chain classes, and kappa and lambda light chain types.

Antisera

The murine monoclonal anti-V_KIIIb antibody has been previously described and characterized (Greenstein *et al.*, 1984). For these studies, ascites containing this antibody were used at a 1:000 dilution. Murine monoclonal anti-kappa antibody (BRL, Baltimore, MD) was from a sodium sulphate precipitate of ascites and was diluted 1:10,000 for use. Anti-TAN, a murine monoclonal antibody in ascites specific for an idiotypic determinant unique to the IgG1-λ cryoglobulin TAN, was diluted 1:1000 and used as a negative antibody control in these experiments.

Affinity purified $F(ab')_2$ of sheep anti-mouse Ig antibody was purchased from Cappel, Inc. (Cochranville, PA) and conjugated via glutaraldehyde with alkaline phosphatase (Sigma Chemical Co.) for use in enzyme-linked immunoassay (ELISA) according to the method of Engvall & Perlman (1972). The enzyme-conjugated antibody was used at 1:100 dilution in the ELISA assays.

$F(ab')_2$ of goat anti-human IgM, IgA, and IgG antibodies (each heavy chain-specific) were purchased from Cappel, Inc.

Assay

Purified proteins or appropriate dilutions of sera were screened for K or V_KIIIb-bearing immunoglobulin by an enzyme-linked immunoassay (ELISA). Two variations were used: in one, the wells of 96-well polyvinyl plates (Costar 2595, Cambridge, MA) were coated by adding 100 μl protein at a final concentration of 100–200 μg/ml in bicarbonate buffer, pH 9·5, and incubating overnight at 4°. For inhibition studies, coating proteins consisted of mixtures of 50μl of an inhibitor protein at 200 μg/ml, and 50 μl of decreasing quantities of the IgG-V_KIIIb reference protein, ALL. After the wells were coated with protein, the plates were washed and post-coated for 1 hr at 37° with 1% bovine serum albumin (BSA) in borate-buffered saline (BBS), rewashed, and incubated for $1\frac{1}{2}$ hr at 37° with either anti-V_KIIIb, anti-K, or anti-TAN in PBS-0·1% Tween-20 (Sigma Chemical Co.) at the dilutions indicated above. The plates were again washed, and incubated for $1\frac{1}{2}$ hr at 37° with affinity-purified $F(ab')_2$ of sheep anti-mouse Ig antibody coupled with alkaline phosphatase. The excess reagents were removed by rinsing and the plates incubated for 45 min at 37° after addition of disodium P-nitrophenyl phosphate (Sigma Chemical Co.) in a diethanolamine buffer. All washes were a series of five rinses with BBS. The optical density (OD) at 405 nm was read using a Dynatech (Alexandria, VA) automated ELISA reader.

In the second method, wells were coated with either affinity-purified $F(ab')_2$ of goat anti-human IgM, or anti-human IgG antibody at 100 μg/ml and post-coated with 1% BSA as described above. Diluted sera or purified protein in PBS-0·1% Tween 20 were added to the wells and the plates incubated for $1\frac{1}{2}$ hr at 37° in order to react antigen with the bound antibody. Further washes and incubations with the alkaline phosphatase-coupled antisera and substrate were identical to those described above. The zero baseline for OD was established using wells which received no test immunoglobulin, but were otherwise treated as the experimental wells. The amount of K or V_KIIIb in experimental sera or proteins was quantified by comparison of their OD for V_KIIIb and K to a standard curve of the optical density for known amounts of the monoclonal IgG-V_KIIIb protein ALL mixed with IgG1-λ, MIT assayed simultaneously and under identical conditions.

RESULTS

Sensitivity of ELISA assay

Preliminary data suggested that, although 10% of the normal serum IgM-K pool comprised V_KIIIb light

chains, IgG appeared to be devoid of this light chain sub-subgroup. However, the limit of sensitivity of the assays used initially was approximately 5% of V_KIIIb. Therefore, assays were developed which routinely detected less than 0·4% V_KIIIb.

The assays made use of the IgG1 myeloma protein ALL which has previously been shown to belong to the V_KIIIb sub-subgroup by primary amino-acid sequence analysis (G.N. Abraham, unpublished results), and by its binding to a murine monoclonal anti-V_KIIIb antibody (Greenstein *et al.*, 1984). Equivalent volumes of ALL in final concentrations ranging from 100 μg/ml to 0·2 μg/ml were mixed with a constant final concentration (100 μg/ml) of three different IgG inhibitor proteins.

The mixtures were either coated directly onto wells of polyvinyl plates (Fig. 1) or incubated on goat anti-human IgG antibody-coated plates (data not shown). As shown in Fig. 1, the ELISA has a limit of sensitivity of about 0·4 μg/ml V_KIIIb in ALL, mixed with 100 μg/ml of either (i) purified normal human IgG (from individual KS), (ii) MIT (IgG1-λ), or (iii) CF II. Since the absorbance for V_KIIIb in ALL at 0·4% (0·4 μg/ml in 100 μg/ml of IgG) is greater than that obtained for either KS-IgG or CF II at concentrations of 100 μg/ml, it is concluded that the IgG-V_KIIIb concentrations in these preparations are less than 0·4%. As a control, the reactivity of anti-TAN antibody with ALL mixed with KS-IgG is virtually undetectable, indicating that ALL does not bind non-specifically to mouse or sheep Ig.

Decreasing amounts of ALL were added to a constant concentration of MIT, KS-IgG, or CF II as carriers and reacted with $F(ab')_2$ of goat anti-human IgG antibody coated onto wells of polyvinyl plates (data not shown). Results similar to those shown in Fig. 1 were obtained. However, 0·2 μg/ml of ALL could be detected in these assays.

These data demonstrate that ELISA is equally sensitive in measuring the V_KIIIb determinant by both direct and indirect antigen binding assays. The data also show that IgG purified from a single individual (KS) or pooled (CF II) normal human sera contain less than 0·4% of IgG-V_KIIIb since, even at concentrations of 100 μg/ml, the optical densities produced by these preparations in both assays were less than those obtained for 0·2–0·4% ALL alone. In order to further confirm this finding, IgG was similarly purified from sera of three additional normal volunteers (JM, CC and ST) and each preparation assayed for IgG-

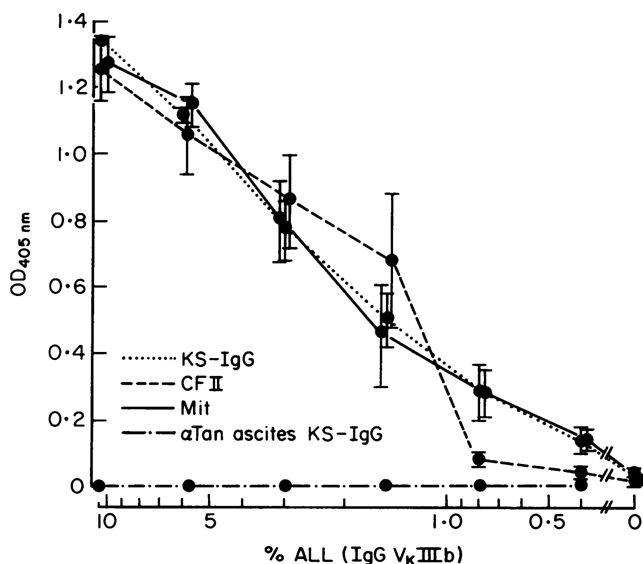

Figure 1. ELISA can detect 0·4 μg/ml ALL in 100 μg ml IgG coated directly on polyvinyl plates. Polyvinyl wells were coated with 100 μl protein (50 μl ALL, 25 μg/ml–0·8 μg/ml mixed with 50 μl of carrier protein at 200 μg/ml) as described in Materials and Methods. In every case, 0·4 μg/ml ALL mixed with either KS-IgG, CF II, or MIT had an OD significantly higher than the three IgG preparations alone (0% ALL) as determined by Student's *t*-test ($P<0·02$, $P=0·05$ and $P<0·001$, respectively). At >11% ALL, OD is >1·5.

Table 1. Purified IgM contains detectable V_KIIIb but purified IgG does not

Anti-isotype coat	Purified Ig	OD anti-V_KIIIb	OD Anti-K
Anti-IgG	IgG	0	$0{\cdot}270 \pm 0{\cdot}087$
Anti-IgG	IgM	NT*	$0{\cdot}026 \pm 0{\cdot}012$
Anti-IgM	IgG	NT	$0{\cdot}033 \pm 0{\cdot}030$
Anti-IgM	IgM	$0{\cdot}377 \pm 0{\cdot}030$	$0{\cdot}948 \pm 0{\cdot}024$

Wells were coated with 100 μg/ml of F(ab′)$_2$ goat anti-human IgM or IgG. IgM and IgG were chromatographically purified as described in Materials and Methods, and the V_KIIIb in each pool was assayed. These data are pooled measurements of levels in preparations from three individuals (JM, CC and ST), mean ± SD.

* NT = not tested.

V_KIIIb. In each instance none was detectable. However, type Kappa light chains were readily quantified in IgG, and column-purified IgM from these same individuals contained readily detectable IgM-K and IgM-V_KIIIb (Table 1). The optical densities obtained for IgM-K were higher than those obtained for IgG-K. This most likely reflects an increased K:λ ratio in IgM compared to IgG (4·6:1 K:λ in IgM versus 2:1 in IgG) (Kabat, 1976).

Absence of V_KIIIb in light chains isolated from pooled IgG

A possible explanation for the lack of detectable V_KIIIb light chains in IgG could be inaccessibility of V_KIIIb determinants in the intact IgG molecule. Steric hindrance or 'hidden' determinants have been suggested as the reason for an inability to produce murine monoclonal anti-V_H region antibodies against intact immunoglobulin (Kubagawa *et al.*, 1982). In order to rule this out, light chains were isolated from CF II by partial reduction and alkylation, and Sephadex G-100 chromatography, and were coated onto wells of polyvinyl plates at 100 μg/ml. The LAR light chain

Table 2. Anti-V_KIIIb does not bind CF II light chains

Light chain source	OD anti-V_KIIIb	OD anti-K
Experiment 1		
CF II light chains	$0{\cdot}004 \pm 0{\cdot}004$	$0{\cdot}356 \pm 0{\cdot}020$
LAR*	$1{\cdot}480 \pm 0{\cdot}030$	$0{\cdot}580 \pm 0{\cdot}250$
Experiment 2		
45 min substrate incubation		
CF II light chains	0	$0{\cdot}491 \pm 0{\cdot}057$
LAR	$0{\cdot}659 \pm 0{\cdot}040$	$0{\cdot}765 \pm 0{\cdot}030$
2 hr substrate incubation		
CF II light chains	0	$1{\cdot}310 \pm 0{\cdot}132$
LAR	> 1·500†	> 1·500†
Experiment 3		
Pooled IgG light chains‡	0	> 1·500†
ALL light chains	$0{\cdot}836 \pm 0{\cdot}208$	> 1·500†

Light chains were coated onto plates at 100 μg/ml and OD for K and V_KIIIb light chains measured as described in Materials and Methods. Data points represent quadruplicate wells (mean ± SD).

* LAR, V_KIIIb-Bence-Jones protein.

† Automated ELISA reader limit of assignment of 1·5 OD units.

‡ Pooled IgG light chains were prepared by reduction and alkylation of IgG purified by G-200 and DEAE chromatography of pooled sodium sulphate precipitates as described in Materials and Methods.

was similarly reduced and alkylyated, and used as a positive control. The conditions of partial reduction and alkylation have been shown not to affect the V_KIIIb determinant (Greenstein *et al.*, 1984). The binding of both anti-K and anti-V_KIIIb were then measured. The data shown in Table 2 demonstrate that the CF II light chains contain significant concentrations of K and retention of the K determinant but no detectable V_KIIIb light chains.

In Experiment 2, the absorbance for CF II light chains was measured both at 45 min and at 2 hr to demonstrate that the OD for V_KIIIb in the CF II light chains would not increase with time. Clearly, the OD for V_KIIIb in CF II light chains remained below the OD for the uncoated wells. Further, these same results were obtained with purified light chains from pooled IgG isolated by the alternative method of Na_2SO_4 precipitation and DEAE chromatography, and using identically purified ALL light chains as a positive control (Experiment 3). Therefore, the IgG serum pool does not contain detectable V_KIIIb light chains.

Table 3. Normal serum IgG and IgA contain no detectable V_KIIIb

Anti-isotype coat	Ig measured	OD Anti-V_KIIIb	OD Anti-K
IgG	IgG	0·037 ± 0·026	0·573 ± 0·034
IgA	IgA	0	0·610 ± 0·035
IgM	IgM	0·365 ± 0·056	0·442 ± 0·075
IgG	0%ALL	0·015 ± 0·014	0·037 ± 0·023
IgG	0·2%ALL	0·100 ± 0·005	0·136 ± 0·010
IgG	100%ALL	0·615 ± 0·020	0·370 ± 0·002

Wells were coated with $F(ab')_2$ of goat anti-human IgG, IgA or IgM antibody followed by incubation with sera diluted 1:20 in PBS-0·1% Tween. Sera were from six individuals (CC, ST, SC, KS, RH and AW), each assayed in triplicate, the data pooled and expressed as mean ± SD.

V_KIIIb values for IgG and IgA are significantly less than for 0·2% ALL ($P < 0·02$ and $P < 0·005$, respectively) and are not significantly different from 0% ALL ($P < 0·2$ for IgG and IgA).

Percentage of V_KIIIb in normal serum IgG-K, IgA-K, and IgM-K

The V_KIIIb and kappa light chains in IgM, IgG and IgA were measured by coating plates with anti-IgM, anti-IgG or anti IgA and assaying sera as described in the Materials and Methods. Standard curves were constructed for binding of anti-V_KIIIb and anti-kappa to ALL diluted in a constant concentration of IgG1-λ MIT as a carrier protein (MIT does not react with either the anti-V_KIIIb or anti-Kappa antisera). In four separate experiments, the correlation coefficient for the binding of anti-K *vs* anti-V_KIIIb was always greater than or equal to 0·92, indicating a linear relationship between the two variables. Therefore, it was possible to directly compare the optical densities obtained for K and V_KIIIb. The ratio of V_KIIIb/K as a percentage of concentration was determined by directly comparing the experimental values to the standard curves for V_KIIIb and K, and then calculating the percentage of V_KIIIb in K.

The optical density readings obtained for IgM, IgG, and IgA-kappa and V_KIIIb are shown in Table 3. Samples from six normal individuals were assayed in triplicate and the data pooled. As noted, the range of values for the optical densities indicate that serum IgG and IgA do not contain quantifiable levels of V_KIIIb light chains. These values are significantly less than those obtained for the ALL reference at a concentration of 0·2%, and are not significantly different from the background. In contrast are the absorbances obtained for the IgG-K (0·573) and IgA-K (0·610). From these values and those obtained for the standard, it is possible to conclude that IgG-V_KIIIb and IgA-V_KIIIb comprise less than 0·2% of the K pool for these immunoglobulins. The experiment was repeated several times and, in each case, the results were the same.

As shown in Table 3, the values obtained for IgM-V_KIIIb were markedly different from IgG-V_KIIIb and IgA-V_KIIIb. A significant quantity of IgM-V_KIIIb was noted. The average percentage of IgM-K which is V_KIIIb-derived from four experiments by assaying serum obtained from the same six individuals was 25·5 ± 15%. It must be noted that the percentages of V_KIIIb in K are independent of serum dilution. That is, over the range of dilutions, 1:2 to 1:1000, the percent remains constant. Further, even at a 1:2 serum dilution, no V_KIIIb was detected in IgG and IgA. Thus, it is apparent from cumulative data that nearly all of the detectable V_KIIIb in human serum is associated with IgM.

DISCUSSION

Previously, it has been demonstrated that the V_KIIIb variable region sub-subgroup was unique in its preferential association with monoclonal IgM autoanti-

bodies, especially cold agglutinins (Capra *et al.*, 1972; Feizi *et al.*, 1976) and rheumatoid factors (Kunkel *et al.*, 1974; Ledford *et al.*, 1983). Serological studies have determined that the incidence of V_KIIIb is 60% among monoclonal IgM anti-IgG cryoglobulins, and 8% among random control monoclonal IgM paraproteins (Kunkel *et al.*, 1974). In the present studies, by assay of fractionated or whole normal human serum, another unique property of the V_KIIIb sub-subgroup has been defined. The light chains containing the V_KIIIb sub-subgroup are detected in an overwhelming abundance in association with the mu heavy chain in six normal individuals, but not with gamma or alpha heavy chains.

Several theories might explain this novel finding of exclusive association of V_KIIIb with mu heavy chain. One explanation is that the V_KIIIb light chain is unable to physically associate with the gamma and alpha heavy chains. This possibility seems unlikely, since at least two myeloma patients with IgG-V_KIIIb monoclonal proteins, LAR and ALL, are known. However, these proteins may be aberrant and not reflective of normal IgG. It has been demonstrated in other systems that a non-random association of heavy and light chains exists (de Preval & Fougereau, 1976; Grey & Mannik, 1965). That is not to say, however, that random association of heavy and light chains does not occur, as mixed molecules are produced by hybridomas (Cotton & Milstein, 1973). There appears to be a requirement for homologous chains to form active molecules (Kranz & Voss, 1981), and it has been suggested that antibody-producing cells must be selective when synthesizing heavy and light chains which can form an antigen-binding site. Perhaps IgG or IgA-V_KIIIb is an unfavourable antigen-binding molecule.

A second explanation involves active suppression of the production of IgG-V_KIIIb and IgA-V_KIIIb by a T suppressor-cell circuit. Polyclonal B-cell activation with both T-dependent and T-independent mitogens has been attempted in our laboratory to determine whether a hypothetical or putative suppression can be abrogated. Preliminary results indicate that suppression is probably not the mechanism of regulation.

A third explanation for this predominant association of V_KIIIb with mu heavy chains is that there is an intracellular block in the switching of B cells from IgM-V_KIIIb to IgG or IgA-V_KIIIb secretors. That is, for unknown reasons, clones of cells which have rearranged their heavy chains to produce mu chains and then rearranged their light chains to produce V_KIIIb are committed to that combination and do not progress to gamma or alpha heavy chain production. A corollary of this theory is that the V_KIIIb light chain is involved in responses which simply do not undergo switching due to the nature of antigens involved in the response and the type of T-cell help involved.

It is well known that a normal antibody response is heterogeneous, involving many clonotypes with distinct idiotype expression and different classes of heavy chain. The results presented here appear to be the first report of an entire sub-subgroup of light chains being restricted in its heavy chain association. Presumably V_KIIIb light chains have a wide spectrum of potential antigenic specificities; therefore, to demonstrate such a high degree of preferential association with one heavy chain is remarkable.

Given the unique properties of the V_KIIIb light chain, that is, abundance in autoantibodies and preferential association with mu heavy chain, it would appear that this light chain variable region subgroup, and the cells which produce it, deserve much closer scrutiny.

ACKNOWLEDGMENTS

The authors thank Dr Julia Greenstein for her helpful discussions and Pearl Gabriel for her invaluable assistance in preparing this manuscript. Grant support was provided by the David A. Welk fund for gammaglobulin research, USPHS research grants AI-21288, AI-11550 and AI-19658, USPHS training grants AI-00028 and GM-07102, the James P. Wilmot Foundation, and program project grant AI-21288.

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