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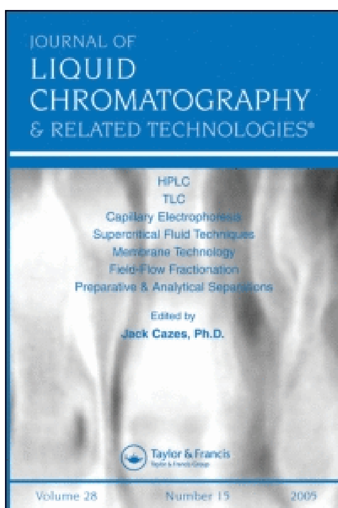

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Comparative Tryptic Peptide Analysis Of Rabbit Heavy Chain Allotypes By Hplc

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COMPARATIVE TRYPTIC PEPTIDE ANALYSIS OF RABBIT HEAVY CHAIN ALLOTYPES BY HPLC

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ABSTRACT

Reversed-phase, high pressure liquid chromatography (HPLC) in a modified TFA/H_2O-TFA/acetonitrile gradient has been successfully applied to the structural analysis of serologically defined rabbit immunoglobulin heavy chains. This mobile phase modification yields a relatively flat baseline at high UV sensitivity. Approximately 40 distinct tryptic peptides were resolved from each heavy chain, representing the V_Ha^+ (a1, a2, and a3) and V_Ha^- (y33,30 and y33,$^-$) immunoglobulin allotypes. About 30 peptides were shown to be derived from the Fc region (C_H2 and C_H3), and 8-10 peptides from the Fd region (V_H1 and C_H1). Seven Fd peptides were shared by all V_Ha^+ and V_Ha^- heavy chains. The a1 and a2 digests each displayed one allotype-specific peptide, whereas no allotype-specific peptides were observed for the a3 heavy chain. No differences were detected between the y33,30 and y33,$^-$ peptides; however, both expressed a common y-specific peptide. Amino acid analysis of purified a1-specific and y-specific peptides indicate that the two peptides are very similar in composition to the predicted first N-terminal tryptic peptides of V_Ha1 and V_Ha^- heavy chains, respectively.

INTRODUCTION

The presence of allotypic markers on the variable (V_H) region of the rabbit immunoglobulin heavy chain (H-chain) presents unique opportunities

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for the study of immunoglobulin (Ig) genetics and regulation. Essentially all (95-98%) rabbit Ig molecules are recognized by anti-allotype antibodies and thus may be assigned to one of the four V_H subgroups (V_Ha, V_Hw, V_Hx, and V_Hy). It has been suggested that these allotypes may serve a physiological role as targets for immunoregulatory T and B cells in much the same way that immunoglobulin idiotypes appear to be regulated (1, 2).

The majority (70-90%) of Ig molecules are the product of the V_Ha subgroup allotypes a1, a2, or a3 (collectively referred to as V_Ha^+) (3-4). The remaining Ig molecules (10-30%), designated V_Ha^-, include one variant of V_Hx (x32), two variants of the V_Hy (y33,30 and y33,$^-$), and two variants of V_Hw (w34 and w35) (5-8).

A number of amino acid sequences of allotype-defined V_Ha^+ (a1, a2, and a3) have been reported. Although a few V_Ha^- sequences have been published, none has been correlated with any of the V_Ha^- subgroups (V_Hw, V_Hx, or V_Hy) or their allotypes. As a result, it is difficult to determine the evolutionary relationship of rabbit subgroups to each other or to the well defined mouse V_H subgroups (9). This problem has been due, in part, to the general unavailability of anti-V_Ha^- antisera and the low level of V_Ha^- molecules in normal rabbits. To facilitate the production of larger quantities of V_Ha^- Ig, we have suppressed the expression of the major subgroup (V_Ha^+) Ig, thereby forcing the expansion of the V_Ha^- Ig populations. In addition we have used allotype-specific antibody to affinity purify two V_Ha^- IgG populations (y33,30 and y33,$^-$) from normal and V_Ha^+-suppressed rabbits. The IgG H-chains were further purified and subjected to biochemical analysis for comparison to the V_Ha^+ allotypes.

HPLC on a reversed-phase support was used to identify allotype-specific and subgroup-specific peptides of the V_Ha and V_Hy allotypes. Amino acid composition analyses indicate that the a1- and y-specific peptides represent the N-terminal 10 and 19 amino acid residues of a1 and V_Hy heavy chains respectively.

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MATERIALS AND METHODS

Immunoglobulin production and purification

The rabbits used for this study are of defined Ig heavy chain haplotype and were bred in our colony at Florida State University. Allotype suppression of the major V_Ha allotypes was carried out by neonatal injection of anti-allotype antisera as described by Horng et al. (10).

Serum samples were taken from two or three rabbits representing each of the various allotypes of the V_Ha and V_Hy subgroups. Each serum sample was processed and analyzed separately. IgG for immunization was purified by standard salt precipitation followed by ion-exchange chromatography (11). Production of antibody to the a1, a2, a3 (4), y33 (6), and y30 (7) allotypes has been previously described.

Allotype-defined Igs were purified by affinity chromatography using columns composed of specific anti-allotype antisera covalently bound to CNBr-activated Sepharose 4B. Contaminating IgM and IgA molecules were removed from the IgG preparation by gel filtration over a Sephadex G-150 column.

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Tryptic digestion and peptide mapping

Pure IgG was separated into H and L-chains as described by Fleichman et al. (12). Fc fragments of IgG were prepared by papain digestion (13). Complete reduction and S-carboxymethylation of the H-chain and the Fc preparation were performed as described by Moss (14).

One mg each of completely reduced and alkylated H-chain and Fc were suspended in 1.0 ml of 0.1 M ammonium bicarbonate, pH 8.5. Digestion was carried out by the addition of TPCK (L-1-tosylamido-2 phenyl chloromethyl ketone)-treated trypsin (Worthington) at a 1% enzyme-protein ratio (w/w); the mixture was incubated for 16 hours at 37°C. Undigested material was removed by centrifugation, and the digests were freeze-dried and stored at -70°C in siliconized glass vials.

High performance liquid chromatography (HPLC) was used for peptide mapping, as described by Fullmer and Wasserman (15). The HPLC system

(Waters Associates, Milford, MA) consisted of two M6000 A pumps, a U6K universal injector, a microprocessor system controller (model 720), a recorder/integrator (model 730), and a C18/Corasil (2 cm x 3.9 mm i.d.) guard column coupled with a μBondapack C18 reverse phase column (30 cm x 3.9 mm i.d.). Column eluates were monitored for absorbance at 214 nm with 0.04 absorbance units full scale (AUFS) using a M450 variable wavelength detector.

All mobile phase solutions were filtered through a Millipore all-glass filter apparatus and 0.45 μm nylon-66 filters (Rainin Instrument Co. Inc., Woburn, MA). Immediately prior to analysis these solutions were degassed with a model B-524 Sonifier (Branson Sonic Power Co., Danbury, CT) under vacuum.

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Samples containing 50 μg of the H-chain peptides (one nM) in 20 μl distilled water (Milli-Q water purification system, Millipore Corporation, Bedford, MA) were eluted at 2ml/min in a linear gradient of 0-50% acetonitrile increasing by 1%/min. Pump A contained 0.1% TFA (Pierce Chemical Co., Rockford, IL) in water, and pump B contained 0.075% TFA in acetonitrile (Burdick and Jackson Laboratories, Muskegon, MI). A reverse program of 5 min to reconstitute the initial conditions was employed. The column was reequilibrated for 10 min between each run and the next.

Determination of amino acid composition

Amino-terminal analysis was attempted by the dansyl chloride method as described by Gray (16). Peptides were hydrolysed at 105°C for 24 h in constant boiling HCl in flame-sealed (under nitrogen) heavy-wall ignition tubes. Amino acid composition analyses of the peptides were performed by derivatization of hydrolized peptides with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (2-ME) to form a highly fluorescent substituted isoindole (17).

A Dionex D-300 Amino Acid System (Dionex Corporation, Sunnyvale, CA) was used for the post-column derivatization and analysis. The system was

equipped with a Gilson Spectra/glo fluorometer (Gilson Medical Electronics, Middleton, WI) containing the OPA excitation (7-60x) and emission (3-73M) filters modified with a 15 1 flow cell. The analyzer was operated with a stainless steel column of DC-5A resin (4 x 170mm) and sodium citrate buffers following standard single-column methodology (buffer A: 0.2N sodium citrate, pH 3.25; buffer B: 0.2N sodium citrate, pH 4.25; buffer C: 1.0N sodium citrate containing 0.8N sodium chloride). The column was regenerated with 0.2N sodium hydroxide following each run and reequilibrated with buffer A prior to next sample injection.

Post column reaction of primary and secondary amines was achieved in a single run by use of a 2-pump/2-reaction coil system (18). The secondary amino acids such as proline were detected after reaction with sodium hypochlorite (17).

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RESULTS AND DISCUSSIONS

The allotypes of the V_Ha^+ subgroup are well characterized serologically and biochemically. The published V_Ha^+ and the V_Ha^- amino acid sequences (compiled in ref. 19) indicate that the 14-16 amino acid differences that correlate with rabbit V_H allotypic determinants are located in framework one (FR1) and framework three (FR3) (20).

The V_Ha^- subgroups have also been serologically characterized but have not been correlated to the few reported V_Ha^- protein or nucleic acid sequences (21-22). Thus we are unable to determine the evolutionary relationships between the rabbit V_H subgroups or their relationship to the extensively studied murine V_H subgroups (9). As a first step in the structural characterization of the V_Ha subgroup we have isolated allotype-defined H-chains of the V_Hy subgroup for peptide and amino acid composition comparsion to V_H a1, a2, and a3 H-chains.

Purified H-chains were subjected to the tryptic digestion and the resulting allotype and subgroup-specific peptides were analyzed by HPLC.

We initially used a 0.1% orthophosphoric acid HPLC buffer (23); however, the high salt concentration interfered with subsequent attempts at amino acid analysis of isolated peptides. Consequently, the present peptide analysis was performed in a volatile TFA-based buffer. It should be noted that in order to maintain a stable baseline at high sensitivity, we found it necessary to vary the percentage of TFA (0.075% and 0.1%) between the two mobile phases. If it was left unadjusted (i.e., 0.1% TFA in both mobile phases), the variability of the refractive index within the detector flow cell eventually forced the baseline off-scale before the run was complete. The column effluents were initially monitored at 214, 254, and 280 nm to determine the optimum wavelength for peptide detection. Monitoring at 214 nm revealed the most peptides and thus was used throughout this study.

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Overall, each H-chain sample yielded approximately 40 peptides. The peptide maps for the two to three samples representing each V_H allotype were found to be essentially identical. The comparison of the peptides obtained from a1, a2, and a3 molecules reveals a unique allotype-specific peptide for the a1 H-chain and another for the a2 H-chain. In contrast, no allotype-specific peptides were detected for a3 allotype (Fig. 1). Analysis of the y33,30 and y33,$^-$ tryptic maps reveal a shared y-specific peptide, but no peptide was found to be unique to either of the y allotypes (Fig. 2). Also, no difference was detected between the peptide maps of V_Hy-subgroup H-chains isolated from normal and allotype-suppressed rabbits.

The differences observed for the V_Ha^+ H-chains are not due to the Fc fragments (Fig. 2), because Fc fragments from both the V_Ha^+ and V_Ha^- molecules used in this study express the same constant region allotypes (d12, e15) and display indistinguishable peptide maps as previously reported (24). Thus, the source of the unique peptides is narrowed to the Fd (V_H and C_H1) region of the H-chains. Since no sequence variations have been found in the constant region of the Fd fragment (25) it is concluded that the observed peptide differences are located in the V_H regions.

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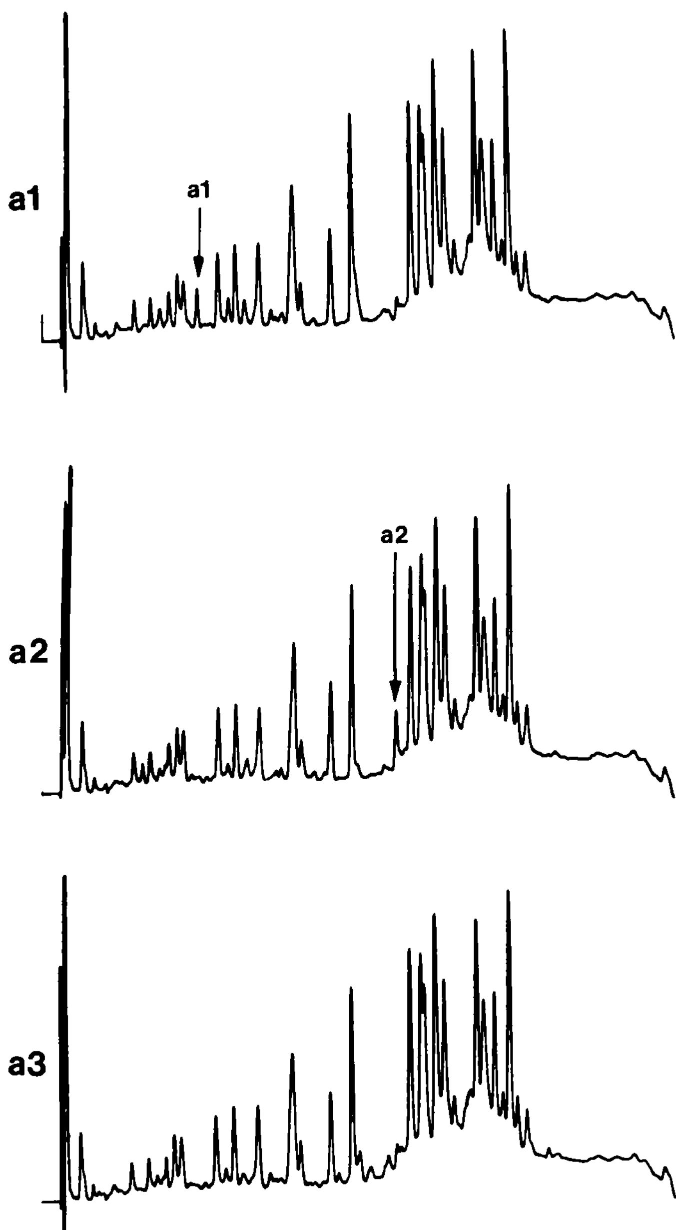

FIGURE 1. Peptide map chromatograms of V_Ha allotype heavy chains (a1, a2, a3). Elution of peptides was achieved in 50 min as described in Materials and Methods. All runs were conducted at room temperature, at wavelength 214 nm. Arrows indicate the allotype-specific peptide identified for each heavy chain.

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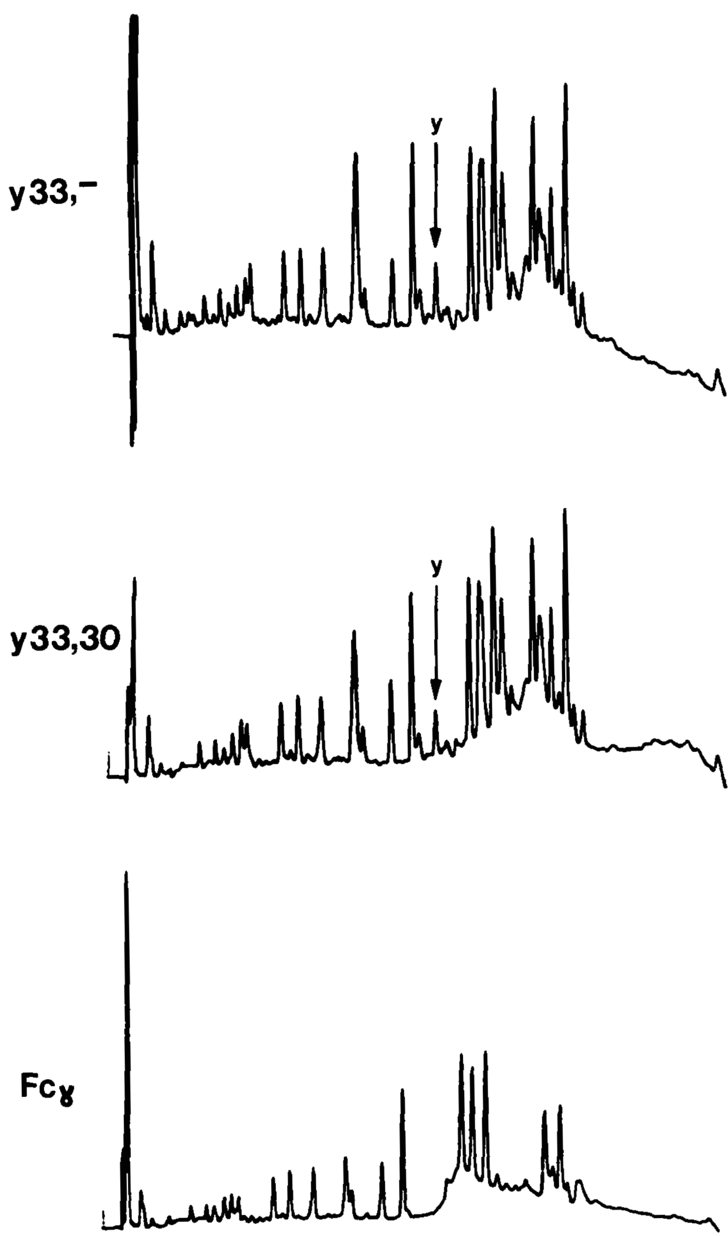

FIGURE 2. Peptide map of V_Hy allotype heavy chains (y33,30, y33,⁻) and Fc fragment. Heavy chains and Fc fragment were incubated with trypsin for 16 h and chromatographed under conditions identical to those described in figure 1.

The number of allotype specific peptides that we have detected is lower than the predicted number of V_H tryptic peptides in those H-chains for which a complete or partial amino acid sequence is known. At least three specific tryptic peptides can be predicted for the V_H framework regions of both the a1 and the a2 H-chains as well as for the pooled (but not allotype or subgroup-defined) V_Ha^- H-chains. The incomplete V_H amino acid sequences currently available for the a3 molecule (18) lead to a prediction of at least one unique peptide at the N-terminal of the V_H region (FR1, residues 1-13). These discrepancies may be explained in several ways. First, it is possible that some peptides coelute with other common peptides of the H-chain and are thus undetectable. Second, it should be noted that all of the mapped H-chains were intentionally derived from normal (unimmunized) rabbits. This approach was used to preclude the detection of fragments resulting from the homogenous hypervariable regions. As a consquence it is likely that some otherwise allotype-specific segments extend for various lengths into a variety of hypervariable segments. This variability in peptide length and composition would result in a large number of very minor (and thus undetectable) peptide populations.

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The available data for FR2 and FR4 sequences for the rabbit Ig reveal a relatively invariant sequence devoid of allotypic-related residues, indicating that the specific peptides are most likely derived from the FR1 and the FR3 regions. Amino acid analyses were performed to localize more precisely the position of our identified allotype and subgroup-specific peptides within the V_H region. The a1-specific and y-specific peptides were isolated and further purified by HPLC (Fig. 3). Attempts were made to determine the N-terminal amino acid sequences; however, because the peptides were resistant to sequencing, we suspect that the fragments have blocked N-terminal residues. Similar results were obtained when intact H-chains were analyzed. Attempts at using the enzyme pyroglutamate aminopeptidase (26) for deblocking the heavy chains were unsuccessful. As

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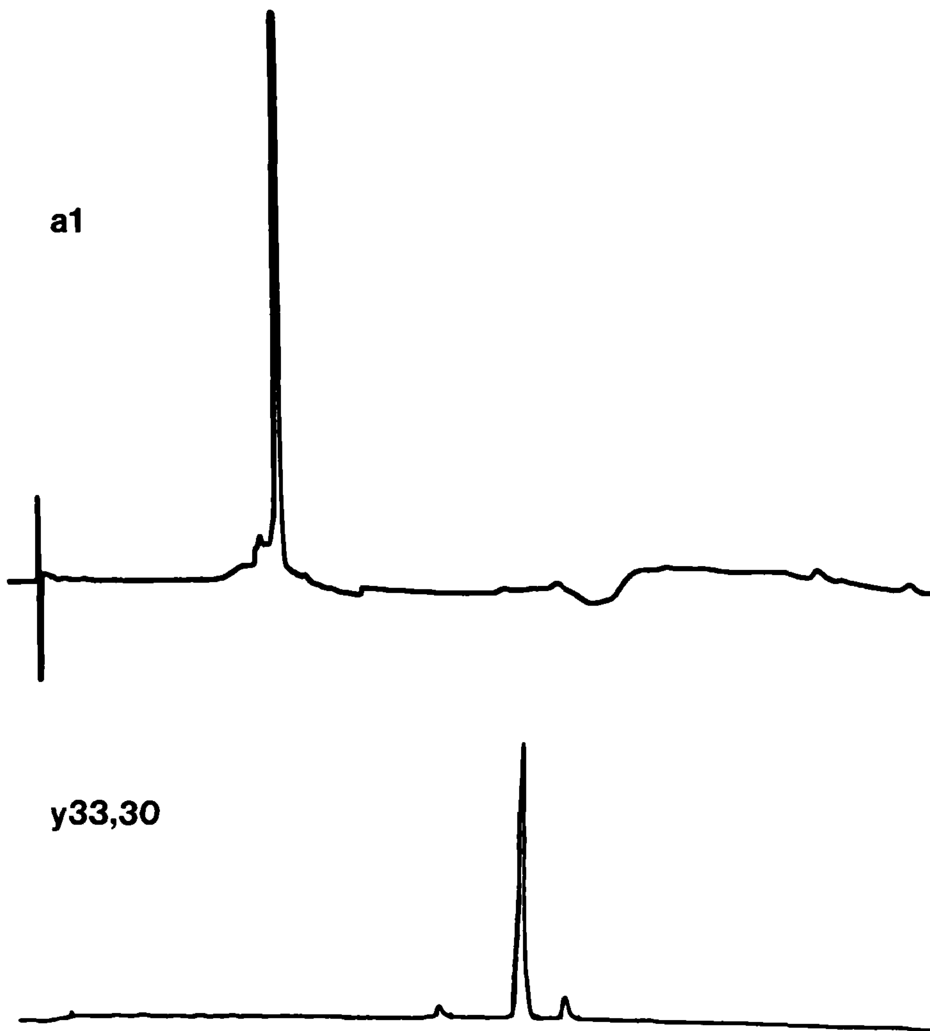

FIGURE 3. Rechromatogram of isolated a1-specific and y-specific peptides. The specific peptides indicated in figures 1 and 2 were isolated and rechromatogramed. The major peaks shown above were reisolated and used for amino acid composition analysis.

an alternative to sequencing, we subjected the a1-specific and y-specific peptides to compositional analysis.

The amino acid composition of the a1-specific peptide was compared to each of the predicted tryptic a1 peptides based on previously reported sequences. The data (Table 1) reveal that the amino acid composition of the a1-specific peptide closely correspond to the predicted tryptic a1 peptide encompassing the first 10 amino acid residues of the V_Ha1 sequences. However, our a1-specific peptide contains three rather than the predicted two glutamic acid residues. This variation in the composition of the a1-specific peptide is not entirely unexpected since several serologically defined subpopulations of the a1 molecule have been described (27).

A comparison of the amino acid composition analysis of the y subgroup-specific peptides from the y33,30 and the y33,$^-$ peptides reveals that each contains identical residues. This finding suggests that allotype-related differences detected in serological assays are located elsewhere in the V_H region. A comparison of the amino acid composition of the y-specific peptide with the previously published (but not serologically characterized) $V_H a^-$ sequences reveals close similarity to a predicted tryptic peptide composed of the N-terminal 19 residues of the V_H region (Table 1). In addition, our y-specific peptide and the published $V_H a^-$ sequences each have a blocked N-terminal amino acid and a lysine residue at the C-terminal end of the tryptic peptide. We detected four glutamic acid residues in the y-specific peptide rather than the five residues in the reported sequences. The three glutamic acid and two glutamine residues of the published $V_H a^-$ sequences appear as glutamic acid under the conditions of our analyses. Similarly, three residues of glysine are detected instead of five glysine residues. It should be noted that one residue of alanine is obtained in the composition of the y-specific peptide. This amino acid is not present in the pooled $V_H a^-$ sequences. Together, these differences suggest that the isolated V_Hy molecules represent a defined subgroup that

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differs from the (one or more) subgroup(s) represented in the previously published V_Ha^- sequences.

The amino acid sequences derived from the sequencing of cloned rabbit germline DNA (20, 22) indicate that at least seven of the first 19 residues of FR1 (positions 5, 6, 8, 11, 13, 15 and 16) do not correspond with previously published amino acid sequences of the V_Ha^- molecules. These data further suggest the existence of subpopulations (subgroups of V_Ha^- molecules). Positions 5, 8, 13, and 16 have been previously shown to be correlated with differences among the three V_Ha allotypes as well (28). Thus, additional sequence studies on H-chains of V_Hw, V_Hx, and V_Hy will be required to elucidate fully the structural correlates of these serologically defined subgroups.

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TABLE 1

Amino Acid Compositions of Peptides Specific for a1, y33,30, and y33,$^-$ *

amino acids	Tryptic Peptides				
	predicted V_Ha^- 1st 19 aa	y33,30 composition	y33,$^-$ composition	predicted a1 1st 10 aa	a1 composition
Asp	-	0.4	0.4	-	0.1
Thr	-	-	-	-	-
Ser	2.0	2.2	2.1	2.0	2.0
Glu $	5.0	3.8	4.3	2.0	3.0
Gly	5.0	2.9	2.8	2.0	2.2
Ala	-	0.9	1.0	-	0.3
Val	1.0	1.0	1.5	1.0	1.2
Met	-	-	-	-	-
Ile	-	-	-	-	-
Leu	3.0	2.8	2.9	#	0.3
Arg	-	-	-	1.0	1.0
Lys	1.0	1.0	1.0	-	-
Tyr	-	-	-	-	-
Phe	-	-	-	-	-
His	-	-	-	-	0.4
Pro	1.0	1.0	1.0	-	-
Cys	-	-	-	-	-
Trp	-	ND	ND	-	ND

* compositions are given as residues/molecule of peptide.
# leu and val at position 4 are interchangeable.
$ glutamine was determined as its respective acid.
ND not determined.

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