CHEMICAL AND IMMUNOCHEMICAL EVIDENCE FOR DIFFERENT CLASSES OF RABBIT LIGHT POLYPEPTIDE CHAINS*

By E. Appella, R. G. Mage, S. Dubiski, and R. A. Reisfeld

NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND, AND DEPARTMENT OF IMMUNOLOGY, THE TORONTO WESTERN HOSPITAL, UNIVERSITY OF TORONTO, CANADA

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Genetically controlled antigenic differences between the immunoglobulins produced by individual rabbits (allotypic differences) are detectable with antisera prepared by isoimmunizations with rabbit immunoglobulins.[1] Allotypic determinants present on immunoglobulin G (IgG) of rabbits are controlled by at least two genetic loci *a* and *b*, which are not closely linked. Allelic genes of the *a* locus, a^1, a^2, and a^3, control structural features of immunoglobulin heavy polypeptide chains, whereas allelic genes of the *b* locus, b^4, b^5, b^6, and b^9, control structural features of the light polypeptide chains.[1, 2]

Oudin has designated as *b-negative* (b^-) a population of immunoglobulin molecules (5–20% of IgG) in rabbits homozygous or heterozygous at the *b* locus, which lack detectable *b* locus allotypic specificity. Work in a number of laboratories has suggested that a population of light polypeptide chains devoid of allotypic specificities controlled by the genes at the *b* locus is indeed produced in normal rabbits. Such chains have not been chemically separated from mixtures of normal light chains. However, it has been possible to suppress the phenotypic expression of b5 allotypic specificity by administration of anti-b5 antibody to developing newborn b5 homozygous rabbits.[3] The IgG produced by the treated animals was then essentially all *b-negative* for several months.

The objective of this study was to isolate the *b-negative* light chains from IgG of such allotype-suppressed rabbits, to characterize them by chemical and immunochemical means, and to compare them with light chains from nonsuppressed rabbits expressing the b5 allotypic specificity.

Materials and Methods.—Rabbits: Heterozygous b^4b^5 females, in which the phenotypic expression of the b^5 gene had been suppressed by injection of the corresponding antibody at birth, were mated with homozygous b^5b^5 males; b5 immunoglobulin remained undetectable in the circulation of the pregnant animals. Newborn rabbits obtained from these matings were given intraperitoneal injections of anti-b5 immune serum. Two of the animals used in the present study were given 4.5 mg of antibody nitrogen during the first 8 days of life; the other two were given 10 mg of antibody nitrogen during the first 20 days of life. Homozygous b^5b^5 animals were identified by their inability to synthesize the b4 immunoglobulin,[3] i.e., weekly bleedings during the first 3 months of life showed a declining concentration of b4 immunoglobulin. In contrast, heterozygous littermates showed an increase in b4 IgG concentration paralleling the increase in IgG attributable to synthesis by the young animals. These animals were then kept until the concentration of passively acquired b4 immunoglobulin decreased to the limit of detection by the gel diffusion method; then they were bled at weekly intervals until they started to recover from suppression which resulted in appearance of b5 in their serum. Sera were collected from four animals between 84 and 200 days of age. The nonsuppressed rabbits used for obtaining serum pools were a^1a^1 b^5b^5 and derived from colonies bred at NIH.

Immunochemical methods: Sera collected from the b5-suppressed homozygotes were tested for b4 and b5 in two laboratories by gel diffusion methods, namely, by single diffusion in gel tubes[4] and radial diffusion in serum-agar plates.[5] In serum samples from

which IgG was isolated, the concentrations of IgG estimated by radial diffusion ranged from 0.8 to 1.6 mg N/ml. Several serum samples had no detectable b4 or b5 and thus contained no more than 1% *b-positive* (b^+) IgG. Most of the sera had less than 20 μg of b5 IgG N, and several had 10–40 μg of b4 IgG N/ml. These contaminants represented 1–2% of the total IgG in all but one serum sample which contained 4–6% *b-positive* IgG. Several of the IgG preparations from *b*-negative sera of allotype-suppressed animals were labeled with I^{125} and tested for reactivity with antiallotype antisera.[6] In a preparation from an a1 homozygous rabbit, 81% of the labeled IgG was shown to react with anti-a1 antiserum, whereas no significant reactivity (<3%) with anti-b5 could be detected. Two other IgG preparations also showed no significant reactivity with anti-b5 in similar tests. A goat antiserum to *b-negative* light chain was prepared with the use of light chains from the serum of a single animal which contained less than 1% *b-positive* IgG. The light chain (0.5 mg) incorporated in complete Freund's adjuvant was injected intramuscularly into multiple sites. An additional 0.5 mg incorporated in Freund's incomplete adjuvant was similarly injected 1 month later, and the goat was bled 8 days afterward.

Isolation and chemical methods: The IgG from either suppressed or nonsuppressed serum pools was prepared by sodium sulfate precipitation followed by chromatography on diethylaminoethyl cellulose as described previously.[7]

Partial reduction with dithiothreitol (0.01 *M*) followed by alkylation with C^{14}-iodoacetic acid (New England Nuclear) or iodoacetamide (0.022 *M*) was performed prior to isolation of the light polypeptide chains by gel filtration on Sephadex G-100 as previously described.[7]

Acrylamide gel electrophoresis was performed at pH 9.4 in Tris-glycine buffer and at 25°C in the absence of urea.[8] The acrylamide concentration used was 5%. In order to isolate and identify peptides which could be assigned to the carboxy-terminal end, b^- light chains, which had been isolated by partial reduction and carboxymethylation of IgG with C^{14}-iodoacetate, were subjected to successive chromatography after digestion of the C^{14}-labeled light chains with trypsin (ratio 1:100 in 0.5% ammonium bicarbonate at 37°C for 12 hr). The tryptic digest was lyophilized, dissolved in 0.05 *M* ammonium bicarbonate, and applied to a Sephadex G-25 column (0.9 × 140 cm). The labeled fractions obtained were pooled, lyophilized, and applied first to a Sephadex G-50 column (1.2 × 165 cm, 0.05 *M* ammonium bicarbonate) and then to a Dowex 50-X2 column (0.9 × 27 cm) developed with a linear gradient of 0.2 to 2 *M* pyridine-acetic acid buffer, pH 3.1–5.0. The carboxy-terminal peptide was identified by the C^{14}-label specifically attached to the S-carboxymethylcysteine of the light-heavy interchain disulfide bridge.

In order to isolate any peptides lacking free α-amino groups which could thus be assigned to the amino-terminal end, a b^- light chain preparation was digested with subtilopeptidase A (Nutritional Biochemical Corporation) with a ratio of 1:20 in 0.5% ammonium bicarbonate at 37°C for about 12 hr. The digest after lyophilization was passed through a column of Dowex 50-X2 (H^+ form) (1 × 15 cm) that had been washed with water. Fractionation of the water eluate was performed by paper electrophoresis in pyridine-acetate buffer, pH 6.5 at 3000 v for 60 min. Peptides were detected on a paper strip with a ninhydrin spray reagent[9] and the starch-iodide reagent for peptide bonds.[10] Peptides were eluted from the paper with 0.05 *N* ammonia. After elution, samples were hydrolyzed with constant boiling HCl (at 110°C for 24 and 72 hr) and analyzed in a Spinco model 644 amino acid analyzer equipped with an accelerated system.[11]

Digestion with carboxypeptidase A (Worthington, lot 1630) was carried out with 5 μg of enzyme in 0.5 ml of bicarbonate buffer at pH 8.2 for 2–3 hr at room temperature. Digestion with leucine aminopeptidase[12] ($C' = 64$ min^{-1}/mgN) was carried out with 2 μg of enzyme in 0.5% ammonium bicarbonate for 1 and 6 hr at room temperature. In each case, after digestion, the reactions were terminated by addition of 100 μl of 0.1 *N* HCl and the peptide samples were applied to the amino acid analyzer.

Results.—Immunochemical characterization: Immunoglobulin G preparations as well as light chains isolated from them behaved similarly upon chromatog-

raphy whether obtained from the sera of rabbits suppressed for b5 allotypic specificity (b^-) or from nonsuppressed b5 rabbits representing a mixture of b5 light chains (b^+) with 5–20 per cent of chains lacking b-locus allotypic specificity (b^-). However, we found distinct differences in immunological, electrophoretic, and structural properties of chains isolated from these genetically similar but phenotypically distinct rabbits. The goat immunized with b^- light chains produced a strongly precipitating antiserum (*ca.* 3.6 mg antibody/ml) with which we demonstrated heterogeneity within the b^- population; we also showed that there was no detectable reaction with the b^+ population from nonsuppressed rabbits. This high degree of specificity for b^- chains was demonstrated by a comparison of precipitability of I^{125}-labeled b^- light chains with b^+ chains (we tested two b5 preparations and one b4). We found that more than 95 per cent of the labeled b^- chains were directly precipitable by the goat antiserum, whereas insignificant amounts (0.7–1.3%) of label were "precipitable" from the b^+ preparations. These three preparations had b^- chains selectively removed by gel filtration of sulfitolyzed b4 or b5 IgG on Sephadex G-100 in the presence of 6 M urea.[13] Light chains prepared in this way still retained immunological reactivity since appropriate anti-b allotype antisera reacted specifically with 85–95 per cent of the labeled molecules.

The goat made a small amount of antibody specific for determinants on the Fc-fragment of IgG heavy chains. This activity could be absorbed in gel diffusion studies by addition of a solution of crystalline Fc-fragment (40 μg N/ml) to the antiserum well prior to addition of antiserum. The absorbed antiserum disclosed at least two precipitin arcs in the gamma region upon immunoelectrophoresis of b^- IgG and of some normal rabbit sera.[14] In agar gel it reacted with the immunizing antigen, other b^- light chains, and with mixtures of b^+ and b^- light chains obtained from nonsuppressed b4 and b5 rabbits.

We found that crossing precipitin arcs formed between the goat antiserum and b^- light chains which were subjected to disc electrophoresis on polyacrylamide gel, an indication that the serum detects at least two antigenically distinct forms of light chains within the b^- population. In contrast, goat antisera to b5 light chains do not develop distinct crossing arcs when reacted against electrophoretically diverse populations of b^+ light chains under identical conditions, although double lines are sometimes observed. Several of our previously prepared goat antisera to normal light chain mixtures from b4 or b5 rabbits contain antibodies reactive with b^- light chains from suppressed rabbits. Acrylamide-gel patterns of partially reduced, carboxamidomethylated b^- light chains showed a considerable degree of electrophoretic heterogeneity since this preparation contained at least seven components, differing, however, markedly from that of normal b5 light chain which somewhat resembles a Gaussian curve.

Chemical Studies.—Carboxyl-terminal peptide: Tryptic digestion of the b^- light-chain preparation and fractionation of the products by successive chromatography on Sephadex G-25, Sephadex G-50, and Dowex 50-X2 (H^+) resolved essentially one labeled peptide (C-1) containing about 60 per cent of the initial radioactivity. Quantitative amino acid analysis gave the following composition: Leu, 1.0; Pro, 1.0; Ala, 2.2; Ser, 2.1; Glu, 1.0; and carboxymethyl-

TABLE 1. *Amino acid composition and sequence of C-terminal peptide isolated from tryptic digests of b^- light chains.*

Methods	Amino Acid Composition of Recovered Peptide (moles/mole peptide)						Amino Acid Sequence Deduced
	CMC	Glu	Ser	Pro	Ala	Leu	
Acid hydrolysis	0.7	1.0	2.1	1.0	2.2	1.0	
Carboxypeptidase A (3 hr)			1.0				( )Ser
Leucine aminopeptidase (1 hr)			0.9			0.6	Ser-Leu( )Ser
Leucine aminopeptidase (6 hr)			1.2		0.5	1.0	Ser-Leu-Ala( )Ser

cysteine, 0.7. Carboxypeptidase A digestion revealed 1 mole of serine. Table 1 summarizes the results and shows the partial amino acid sequence obtained. Further evidence for the C-terminal position of C-1 comes from the observation that it lacks both lysine and arginine. Additional proof for its position is the finding that carboxy-terminal serine was demonstrated by carboxypeptidase A digestion in a yield of 0.6 mole/mole of 22,000 gm in two different b^- light-chain preparations. An attempt was made to detect the peptide analogous to the C-terminal peptide (C-1) of b^- chains in light chains carboxymethylated with C^{14}-iodoacetate and isolated from IgG of nonsuppressed b5 rabbits; we detected a labeled peptide which had the same R_f as C-1 upon high-voltage electrophoresis (pH 6.5) of a tryptic digest of these C^{14}-labeled light chains.

Amino-terminal peptide: In order to investigate the amino-terminal portion of the b^- light chains, preliminary experiments were conducted by means of the phenylisothiocyanate method.[15] Three preparations were analyzed by the use of either partially or fully carboxymethylated proteins. A single amino acid, alanine, was detected (0.05–0.1 mole/mole of 22,000 gm), an indication that with this reagent the amino terminal is largely unavailable. An attempt was made to isolate peptides without a free α-amino group by passing a subtilopeptidase digest through a column of Dowex 50-X2 (H^+ form). After concentration of the water eluate and fractionation by electrophoresis on paper at pH 6.5, two peptide spots that stained with the peptide bond reagent but were ninhydrin-negative were found. The first peptide (S1) obtained in a yield of about 40 per cent contained (after acid hydrolysis) glutamic acid, proline, valine, leucine, threonine in molar proportions of 1:1:1:1:0.8. Carboxypeptidase A digestion for three hours released 1 mole of leucine. A second peptide (S2) was also isolated in a yield of about 10 per cent. Amino acid analysis indicated glutamic acid and leucine in the molar proportions of 3:1. Table 2 summarizes the results and shows the amino acid composition obtained. The presence of glutamic acid in both peptides suggests that the absence of a free α-amino group may be due to a terminal pyrrolidone carboxylic acid residue. The interpretation of the finding of the S2 tetrapeptide remains uncertain at the moment. This peptide could represent the amino-terminal sequence of a variant of the b^- suppressed light chain; however, further work is required to ascertain this. An attempt was made to isolate the amino-terminal peptide from the light chains of nonsuppressed b5 rabbits. When a partially carboxymethylated preparation was

TABLE 2. *Amino-terminal peptides isolated from light chains of suppressed and nonsuppressed rabbits.*

Material	Peptides	Mobility*	Amino Acid Composition (moles/mole peptide)	C-terminal of N-terminal peptide
Light chains from suppressed rabbits	S1	−0.43	$Glu_{1.0}Leu_{1.0}Pro_{1.0}Val_{1.0}Thr_{0.8}$	Leu
Light chains from suppressed rabbits	S2	−0.77	$Glu_{3.2}Leu_{1.0}$	
Light chains from nonsuppressed rabbits	S1	−0.58	$Glu_{1.7}Leu_{1.0}Val_{1.2}Thr_{1.0}$	Leu

* Mobility calculated after paper electrophoresis at pH 6.5, compared with glutamic acid with an assigned mobility of −1.0.

passed through a column of Dowex 50-X2 (H^+) and fractionated on paper electrophoresis at pH 6.5, a ninhydrin-negative peptide was isolated. The yield was low (5%); this is reasonable, however, if the peptide was fractionated entirely from the b^- light-chain population which ranges from 5 to 20 per cent. After acid hydrolysis the composition of the ninhydrin-negative peptide showed glutamic acid, leucine, valine and threonine in the molar proportion of 1.7:1:1.2:1. Carboxypeptidase A digestion released 1 mole of leucine. Table 3 summarizes our N- and C-terminal data and compares them with known sequences of chains from myeloma proteins of man and mouse. Considerable homology is evident between λ chains of both species and b^- light chains from allotype-suppressed rabbits.

Discussion.—This study demonstrates that light chains can be isolated from rabbits suppressed for the total expression of b5 allotypic specificity. These b^- light chains lacked detectable *b*-locus allotypic specificity and had immunological and electrophoretic properties distinct from b^+ light chains. Chemical characterization of b^- chains indicated that the N-terminal amino acid is essentially nonavailable, which may be due to the presence of pyrrolidone carboxylic acid (Glp). In this regard, it is of interest that rabbit heavy chains as well as myeloma λ chains in man and mouse, and in isolated cases κ chains in mouse, possess Glp as the N-terminal amino acid. The significance of this is still somewhat obscure, but pyrrolidone carboxylic acids may play a role in polypeptide chain initiation.[21]

A single C-terminal amino acid (serine) was demonstrated in the b^- light-chain preparation, and a single C-terminal peptide was isolated in good yield. Com-

TABLE 3. *Comparison of N- and C-terminal sequences of λ chains from human and mouse myeloma proteins with b-negative rabbit light chains.*

	N-terminal
Rabbit b^-	Glp-Thr(Val, Pro)Leu
Mouse λ (see refs. 16 and 17)	Glp-Ala- Val- Val- Thr- Gln-Gln-Ser- -Ala- Leu-
Human λ (see refs. 18–20)	Glp-Ser- Val- Leu-Thr- Glu-Pro-Pro -Ser- Ala- Ala Ala Asx Ala Ala Val Glu

	C-terminal
Rabbit b^-	-Ser- Leu-Ala(Pro, Ala, Glu, Cys)Ser
Mouse λ (see refs. 16 and 17)	Lys-Ser- Leu-Ser- Arg- Ala- Asp -Cys-Ser
Human λ (see refs. 18–20)	Lys-Thr-Val- Ala- Pro- Thr-Glu- -Cys-Ser

parison of our N- and C-terminal data with the known sequences of λ chains of mouse and man (Table 3) revealed a remarkable homology with the λ chains of both species, suggesting that our b^- preparations could represent populations of rabbit λ chains. This hypothesis is strengthened by preliminary evidence from amino acid analyses, suggesting that b^- chains also possess five half-cystine residues analogous to λ chains in mouse and man, whereas b^+ chains contain seven half-cystine residues. In both man and mouse, κ- and λ-type light chains are antigenically distinct. In an analogous manner the b^+ and b^- rabbit polypeptide chains can also be differentiated by a specific antiserum.

Electrophoretic and immunological heterogeneity of b^- light chains was indicated by multiple bands and by at least two crossing precipitin arcs observed after disc electrophoresis and immunodiffusion. Further evidence of heterogeneity is our finding of two distinct N-terminal peptides from the b^- light chains and a variant one from b^+ nonsuppressed light chains. The presence of different N-terminal peptides could be due to several light-chain subclasses within the b^- population. These subclasses could represent either allotypic variants or the products of related nonallelic cistrons, possibly analogous to the subclasses of human κ chains which have been revealed by sequence studies.[22, 23] In this regard, a number of allotypic specificities reported by Dray, Young, and Gerald[24] included c7 (P), determined by an allele at a locus different from *a* and *b*. Results obtained thus far with b^- light chains indicate a relationship of the *c* locus to some of the b^- chains. A test for genetic linkage of the b^+ and b^- light chains thus becomes possible by the use of these antigenic markers.

The question arises as to whether the b^- population in the suppressed rabbit is representative of the usual spectra of b^- chains produced normally by nonsuppressed rabbits. Suppression of one b^+ allele in heterozygotes at the *b* locus results in a prolonged alteration of relative expression of b^+ allelic light-chain types. In the rabbits described here, the expression of b^+ has been almost totally suppressed; this, in turn, may result in an altered relative expression of possible b^- subclasses. Nevertheless, the group of b^- chains isolated from these developing, young rabbits is undoubtedly more representative of the products of normal functional antibody-producing cells than the myeloma proteins which are the products of plasma cell tumors.

Summary.—The phenotypic expression of b5 allotypic specificity was suppressed by administration of anti-b5 antibody to newborn b5 homozygous rabbits. Treated animals for a limited time produced IgG, essentially all of which lacked *b*-locus allotypic specificity. Thus it was possible to isolate for the first time a class of b^- light chains which could be differentiated by immunological and chemical means. Structural studies of the C- and N-terminal regions of these b^- chains revealed considerable homology with myeloma λ chains of man and mouse. These findings suggest that b^- light chains isolated from allotype-suppressed rabbits may represent a rabbit λ-chain class.

Note added in proof: In a single experiment, peptide S1 has been digested with pyrrolidonylpeptidase (a gift from Dr. R. Doolittle, *Biochemistry*, **7**, 516 (1968)). Subsequently, a neutral, ninhydrin-positive peptide was isolated from this digest by high-voltage electrophoresis at pH 6.5. This peptide revealed after a first-cycle Edman degrada-

tion the absence of threonine and the presence of molar quantities of valine, leucine, and proline, suggesting the sequence of S1 to be Glp-Thr (Val,Pro) Leu.

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