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Genetic Characterization of a New Allele of the Rabbit Group b C_κ Allotypes

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Abstract. An inherited variant ($b4^v$) of the rabbit kappa-chain allotype b4 is characterized by the presence of serine in place of alanine at position 121 and leucine in place of glutamine at position 124. The variant was traced through eight generations of a pedigreed rabbit family. Genetic analysis of this trait demonstrated that it is an allele of the other group b allotypes, and recent breedings have produced rabbits homozygous for this light-chain type. Two findings, other than the amino acid sequence differences, distinguish $b4^v$ from b4 in our colony. First, the level of expression of $b4^v$ in heterozygous rabbits is less than that of b4. For example, in $b4b4^v$ rabbits, approximately 30% of the preimmune IgG carries $b4^v$. In $b4^vb5$ animals, 46% of the IgG carries the allotype b5, although in animals of allotype b4b5, 38% of the IgG is b5. Second, retrospective analysis of some litters revealed an abnormally low frequency of $b4^v$ in male heterozygotes. However, male $b4^vb4^v$ homozygotes were found at the expected frequency in prospective crosses between $b4^vb5$ rabbits.

Introduction

The group b allotypes of the *C* region of rabbit kappa L chains (Oudin 1960a, b, Stemke 1964, Kindt 1975), were among the first discovered immunoglobulin allotypes, and have been well studied both structurally and serologically. Four alleles (b4, b5, b6, and b9) have been identified previously and the amino acid sequence differences among them have been partially defined. Serologic evidence indicates that the allotypic determinants are limited to the C_κ region (Kindt *et al.* 1972). Complete sequences are available for b4 (Chen *et al.* 1974) and b9 (Farnsworth *et al.* 1976); partial sequences are known for the other two alleles (Strosberg 1977). The differences in sequence among the alleles are large; b4 and b9 differ at thirty-three out of one hundred *C*-region positions and a number of insertions and deletions are required to maintain the chains in alignment. Minimal sequence heterogeneity has been reported for the *C* regions of light chains of any one allotype (Kindt 1975).

A previous publication (Sogn and Kindt 1976) described an inherited sequence variant of b4, which was designated $b4^{var}$ [1]. This trait was present in five generations

[1] For reasons of simplicity the variant will be referred to here as $b4^v$

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of a selectively bred rabbit colony but was not found in a homozygous state by retrospective genetic analysis or in combination with any allotype other than b4. Thus, the earlier data were not sufficient to determine whether $b4^v$ was the product of another allele at the *b* locus or of a gene closely linked to that coding for the allotype b4.

This report describes the results of retrospective typing for all litters within our colony which might have received the $b4^v$ trait, and documents statistical analysis of the inheritance pattern. Prospective breedings carried out to pair $b4^v$ with allotypes other than b4 have eliminated the possibility that $b4^v$ is encoded on the same chromosome as b4 and have produced $b^{4v}b^{4v}$ animals. Analysis of the retrospective inheritance data revealed an unexpected pattern of sex association for $b4^v$, with significantly fewer males inheriting the trait than predicted by random assortment. Such bias was not found in the latest generation, which included $b^{4v}b^{4v}$ rabbits. Quantitative analysis for $b4^v$ placed this allotype in the group b allotype 'pecking order' (Dubiski 1972) at approximately the same level as b5, a result consistent with quantitative sequence data for $b4b4^v$ rabbits reported earlier (Sogn and Kindt 1976).

Materials and Methods

Rabbits. The pedigreed rabbits used in this study were from a colony begun at The Rockefeller University by Dr. R.M. Krause and were primarily derived from New Zealand Red rabbits. They possessed only light-chain allotype b4, but all three group a allotypes were represented. The male rabbits mated with the putative b^4b^{4v} female rabbits were a gift from Dr. Kitty Smith (NIH Veterinary Resource Branch) and came from an allotype-defined line (a^2a^2/b^5b^5) with an inbreeding coefficient of approximately 0.45.

Serologic Determination of Allotypes. Allotypic antisera were prepared according to the principles of Oudin (1966) using methods previously described (Kindt 1975). Group a and b allotypes were qualitatively determined by inhibition-of-binding radioimmune assays as previously described (Gottlieb *et al.* 1975). Allotype ratios (b4/b5) were determined by quantitative binding radioimmune assays (Gottlieb *et al.* 1975).

Typing for $b4^v$. Chemical typing of $b4^v$ was carried out as previously described (Sogn and Kindt 1976), by acid cleavage of mildly reduced and alkylated, succinylated L chains, followed by fifteen steps of automated Edman degradation using a Beckman Model 890C Sequencer. Two features of this assay procedure were examined in greater detail for the purposes of the present study.

First, because it was necessary to type for $b4^v$ in rabbits with allotype b4b5 or $b4^vb5$, the contribution of b5 L chains to the sequences observed after acid cleavage had to be determined. This was done by subjecting mildly reduced and alkylated L chains from the b^5b^5 rabbits used in this study to succinylation, and then treating them under acid cleavage conditions. No new amino terminal amino acids were detected in these preparations by sequence analysis. It was further demonstrated that sequence results on L chains from b4b5 or $b4^vb5$ rabbits were not affected by passage of the serum through anti-b5 immunoabsorbent columns prior to the isolation of L chains. These results showed that the presence of b5 L chains in no way affects the chemical determination of b4 or $b4^v$.

In a second experiment, to determine whether $b4^v$ could be assayed without prior separation of H and L chains, H chains were subjected to conditions used for $b4^v$ typing. A pool of H chains obtained from a large number of $b4^v$ typings was succinylated, subjected to acid cleavage conditions, dialyzed, and analyzed by automated sequence analysis. A single major sequence, Pro–Glu–Val–Gln–Phe, was obtained for the five steps analyzed. This sequence matches the known sequence of rabbit H chains for residues 271 through 275 (Hill *et al.* 1966), indicating that the aspartyl–proline bond at positions 270–271 has acid lability similar to that of the aspartyl–proline bond at positions 109–110 of b4 and b9 kappa L chains (Fraser *et al.* 1972). Because of this H-chain acid lability, $b4^v$ could not be determined without prior separation of H and L chains.

Immunoadsorbent Preparation. A column containing specific anti-b5 antibodies was prepared as follows: Anti-b5 serum (20 ml) was passed through a column prepared by coupling 100 mg of pooled

b5 IgG to 40 ml of Sepharose 4B. This column was washed with phosphate-buffered saline, pH 7.0, and the anti-b5 antibodies were recovered by elution with 3 M NH_4SCN in phosphate-buffered saline, pH 7.0. To remove any cross-reactive or denatured antibodies, the eluted material was extensively dialyzed against phosphate-buffered saline, pH 7.0, and then passed through a second Sepharose immunoadsorbent column, to which IgG of all allotypes except b5 was coupled. The specific anti-b5 antibodies were then coupled to Sepharose by the CNBr reaction of Cuatrecasas (1972).

Immunoglobulin Levels. IgG was isolated by passage through columns of DEAE-cellulose in 0.03 M potassium phosphate buffer, pH 7.0. Column fractions were checked for contamination with β-globulins by microzone electrophoresis. IgG was quantitated by absorbance at 280 nm, assuming $A_{280}^{1\%} = 15$.

Results

Complete Pedigree of b4^v Family

The complete pedigree of the family in which b4^v was identified is shown in Figure 1. The chemical typing method previously described for b4^v was used in this study. Appropriate control experiments indicated that b5 L chains do not interfere with

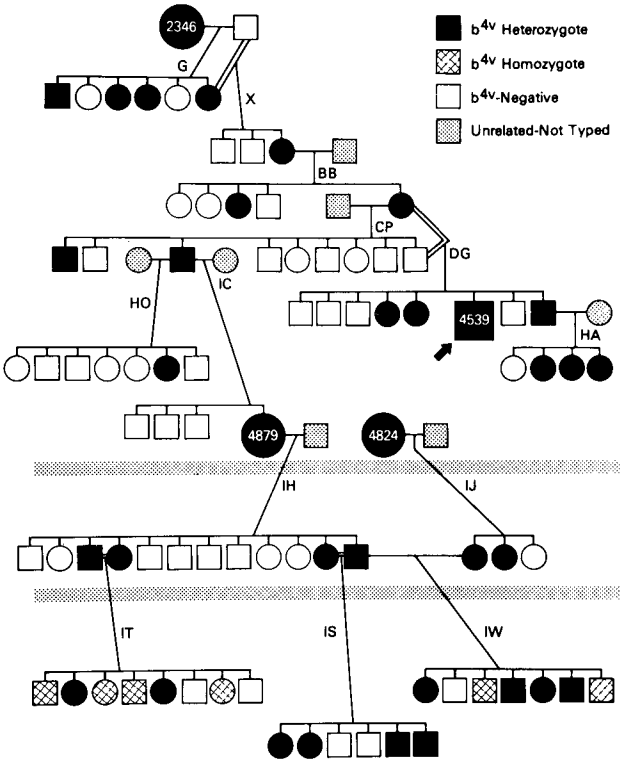

Fig. 1. Pedigree of rabbit family in which b4^v was discovered and propogated. *Letters* represent litter designation. *Squares* represent males and *circles* represent females. *Double lines* indicate consanguinous breedings. *Shaded areas* delineate periods of breeding between rabbits of types b^4b^4 and b^4b^{4v} (*top*), b^5b^5 and b^4b^{4v} (*middle*) and $b^{4v}b^5$ and $b^{4v}b^5$ (*bottom*)

typing for b4 or $b4^v$. Further experiments demonstrated that typing for b4 or $b4^v$ requires prior separation of H and L chains, because H chains also have an acid labile Asp–Pro linkage.

Retrospective analysis beginning with the proband rabbit 4539 revealed that the trait entered our colony with rabbit 2346, which was purchased at the Carver Rabbitry (Somerville, New Jersey) in 1966. Complete typing of the eight litters (*G*, *X*, *BB*, *CP*, *DG*, *HA*, *HO*, *IC*) produced by mating $b4b4^v$ rabbits (no homozygous $b4^v$ rabbits were identified in retrospective studies) led to identification of the $b4^v$ trait in 18 of 46 progeny. Three $b4^v$-positive rabbits were alive when the typing was done and all were females (4782, 4824, and 4879). Because $b4^v$ was always found in rabbits phenotypically $b4b4^v$, it was not possible to determine from the data above whether $b4^v$ represented a new allele or whether b4 and $b4^v$ were encoded by duplicated and closely linked genes. To test this, rabbits 4824 and 4879 were bred to males of genotype b^5b^5, producing litters *IJ* and *IH* respectively. All of the offspring in these litters were typed as b4b5 by inhibition-of-binding radioimmunoassay. By sequence analysis, six of the 15 rabbits contained the $b4^v$ L-chain sequence (Ser at 121, Leu at 124) with no detectable level of the b4 sequence (Ala at 121, Gln at 124). The other nine gave sequences of the b4 type with no detectable level of the $b4^v$ sequence. Crosses between $b^{4v}b^5$ rabbits yielded six homozygous $b4^v$ rabbits from among 21 offspring. These data clearly indicate that $b4^v$ is inherited as an allele of the b allotype.

Sex Distribution of $b4^v$

The pedigrees for ten complete litters with one parent apparently heterozygous for $b4^v$ are included among those in Figure 1. Based on the hypothesis that $b4^v$ is an autosomal allele of the other group b allotypes, it would be expected that half of the male and half of the female offspring would inherit the $b4^v$ trait. Table 1 illustrates that this is not the case. The sex distribution of offspring (31 female, 30 male) is

Table 1. Sex Distribution of the $b4^v$ Allotype

	Total Inheritance			Male Inheritance		
	+	–	Total	+	–	Total
Females	17	14	31	5	4	9
Males	7	23	30	0	6	6
Total	24	37	—	5	10	—

normal (Table 1). Approximately half of the females were positive for $b4^v$ but only seven of 30 males were positive. Thus, by the χ^2 test, sex and $b4^v$ do not randomly assort in this group ($P < 0.025$). The skewing in the distribution is largely due to the fact that significantly fewer of the males inherited $b4^v$ than was expected for an autosomal codominant trait. It may further be noted that of six male offspring in the three litters sired by $b4^vb4$ males, none was $b4^v$-positive (Table 1, $P < 0.05$). This abnormal distribution was not observed in the most recent generation (litters *IS*, *IT*, and *IW*), when $b^{4v}b^5$ rabbits were bred to one another. These crosses yielded six homozygous $b^{4v}b^{4v}$ offspring, four of which were males.

Allotype Selection in b4b5 and b4^{v}b5 Progeny

The relative levels of b4 and b5 in IgG from six-month-old rabbits were assessed by quantitative radioimmunoassay. Results obtained for six rabbits of allotype b4^{v}b5 and six control-litter mates of allotype b4b5 are shown in Table 2. The two groups are significantly different at the 95% confidence level, while the control group is not significantly different from a group of adult b4b5 rabbits examined previously (Sogn *et al.* 1977). Immunoglobulin levels in four-month-old b4^{v}b4^v rabbits were 2.9 ± 0.5 mg/ml, which is not different from levels observed in control-litter mates of genotype b^5b^5 (3.1 ± 0.4 mg/ml).

Table 2. Allotype Selection in b4b5 and b4^{v}b5 Progeny

% b4		
b4b5 adults	b4b5 (6 months)	b4^{v}b5 (6 months)
70.2	65.2	57.2
50.3	61.9	54.5
75.0	62.4	59.1
77.6	57.4	51.4
71.7	61.8	49.9
68.8	65.1	51.9
62.4		
68.0 ± 7.1 (Mean ± 2σ)	62.3 ± 2.3 (Mean ± 2σ)	54.0 ± 2.9 (Mean ± 2σ)

Discussion

This study documents results of inheritance studies on b4^v, a structural variant of the rabbit C_κ region. The inheritance pattern of b4^v indicates that it is an allele of the other b allotypes (b4, b5, b6, and b9) and recent breedings have produced rabbits homozygous for this trait. It is distinguished from the other, structurally diverse b allotypes because of its close structural and serological relationship to b4. From the data presented here, the only criterion lacking for calling b4^v a new group b allotype by the classical definition is an antigenic distinction between b4 and b4^v. Evidence for such a distinction has been found in collaboration with another laboratory and will be reported soon (Smith *et al.* 1978). Thus b4^v represents the fifth allele reported for the *b* locus.

In addition to at least two amino acid interchanges, b4^v is distinguishable from b4 in two ways. First, it can be assigned a position in the b allotype 'pecking order' (Dubiski 1972) significantly below b4 and approximately level with b5. Immunoglobulin with b4^v L chains constitutes 54% of total b allotype positive IgG in b4^{v}b5 rabbits, but only approximately 30% in b4b4^v rabbits. Because of the minimal structural and serologic differences between b4 and b4^v, this difference in relative levels of expression is most likely due to a combination of significantly different V_κ repertoires and predominantly cis synthesis.

The second difference between b4 and b4^v concerns the sex distribution that became apparent upon completion of retrospective typings. For all litters with one

parent negative for b4^v and the other parent a b4^v heterozygote, only seven of 30 male offspring were b4^v positive (Table 1). This indicates that, in these litters, sex and b4^v did not randomly assort ($P < 0.025$). Because the autosomal nature of the group b allotypes is firmly established (Oudin 1960a, b), prospective breedings were carried out and offspring examined for sex-linkage of b4^v. In litters from matings between b4^{v}b5 rabbits, no unusual sex distribution was apparent. Thus, earlier results might reflect chance linkage, in some of the rabbits, between b4^v and a functionally unrelated trait which was lethal in males. The probability of this is lessened by the fact that the number of males weaned from these litters was no different than that of females.

The genetic behavior of b4^v unfortunately provides only a measure of negative evidence about the C_κ genes of the rabbit. The number, arrangement, and control of expression of these genes remain in doubt. It has been suggested, solely on the basis of large structural differences, that the structural genes encoding the group b allotypes (as well as those encoding other complex allotypes) are all present on the same chromosome, with an apparent allelic nature conferred by an allelic regulatory gene (Bodmer 1973, Farnsworth *et al.* 1976). Reports of latent group b allotypes (Strosberg *et al.* 1974, Yarmush and Kindt 1978, Francis and Mandy, unpublished results) provide experimental support for this suggestion.

For any single allotype, the number of gene copies is also not known. Precedents exist for multiple copies of C_L genes in the rabbit and in other species. Isotypes of lambda L chains are well characterized in the human (Ein 1968, Gibson *et al.* 1971, Hess *et al.* 1971), and genetic evidence suggests their existence in the rabbit (Gilman-Sachs *et al.* 1969). Presumptive evidence exists for C_κ isotypic variation in the LOU rat (Starace and Querinjean 1975, Wang *et al.* 1976). Minor structural (Kindt 1975) and serological (Thunberg 1974, van der Loo *et al.* 1975) variation has been noted among rabbit κ L chains of a single group b allotype, although none of the variants has been characterized genetically.

If, as seems likely, b4^v originated by mutation from b4 after speciation of the rabbit, then b4^v is a potential probe for the number of *b4* genes in the rabbit. Because no positive indication for multiple C_κ genes was obtained in the study of this allotype, resolution of the question must come from one of two other areas of study. One of these is the evolutionary record. It is known that other lagomorphs have C_κ determinants that are cross-reactive with the group b allotypes (Landucci-Tosi *et al.* 1973, Cazenave and Roland 1976, van der Loo *et al.* 1976, Smith and Mandy 1978). In many instances, single L chains bear determinants that are cross-reactive with more than one of the allotypes. Unfortunately, without structural data, these intriguing findings are difficult to interpret. A promising area of investigation, in light of recent technical advances, is RNA-DNA hybridization and DNA sequencing. These molecular biological techniques have the potential to completely define the genetic arrangement at the group b locus. After this is done, further examination of the biochemical mechanisms controlling expression of these genes will be possible.

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