A GENETIC POLYMORPHISM IN THE CONSTANT REGION OF RABBIT b4 KAPPA CHAINS*

BY JOHN A. SOGN† AND THOMAS J. KINDT

(From The Rockefeller University, New York 10021)

Much of what is known about the number and arrangement of genes encoding immunoglobulins, and the mechanisms involved in their selective expression, derives from studies involving allotypes. Rabbit allotypes have figured prominently in these studies particularly because they are found in almost every region of the immunoglobulin molecule. Amino acid sequence studies on rabbit allotypes of group a in the V_H region and group b in the C_\kappa region have revealed an unusual degree of structural complexity within these allotypes in that many amino acid differences are found between alternative forms (1). For example, the b4 kappa-chain sequence differs in the constant region from that of the b9 kappa chain at approximately 33% of the positions (2). A further complexity of the rabbit C_\kappa region is that b4 light chains from homogeneous antibodies differ from one another in certain positions (3, 4).

The present report describes an inherited C_\kappa-region structural variant observed among L chains with the allotype b4. This variation of allotype b4 (b4var) consists of two amino acid substitutions, at residues 121 and 124. The variation has been observed in five generations of a pedigreed family. Serologic analysis has revealed that b4 var chains are indistinguishable with regard to their b4 allotype from those with the prototype b4 sequence. Use of this variant as a genetic marker can provide information concerning the number of genes encoding light-chain constant regions.

Materials and Methods

Rabbit 4539 (ala3/b4b4) underwent three immunization series with Group C streptococcal vaccine using methods previously described (5). During the third series, a monoclonal response was observed and three nonsurgical exchange transfusions (6) were carried out. A homogeneous antibody with allotype a3b4 (4539 Ab) was isolated from the transfusion plasma by DEAE-cellulose chromatography and chromatography on a Group C carbohydrate immunoabsorbent column (7). Amino acid sequence studies on the light chain of 4539 Ab have been carried out using a strategy described elsewhere (8). Constant-region sequence variation was first observed upon sequence determination of a peptide (residues 110-207) isolated after acid cleavage (9) of the tryptic fragment encompassing residues 62-207.

Analysis of rabbit sera for the presence of this variant sequence was carried out as follows: IgG, isolated by DEAE-cellulose chromatography, was mildly reduced and alkylated. Heavy and light

* Supported by U. S. Public Health Service grants NIAID AI08429 and AI11439, and by a Grant-in-Aid from the American Heart Association, 75-812.
† Fellow of the Arthritis Foundation.

† Abbreviations used in this paper: 4539 Ab, antibody with allotype a3b4; b4var, variation of allotype b4; PTH, phenylthiohydantoin.
chains were separated by chromatography on a column of Sephadex G-75 in 10% acetic acid (10). The light-chain fraction was succinylated to irreversibly block the amino terminus and all lysine residues and the aspartyl-proline bond between residues 109 and 110 was specifically cleaved by the acid treatment described by Fraser et al. (9). This procedure involves incubation of the light chains for 5 days at 40°C at a concentration of 1 mg/ml in 7 M guanidine-HCl made 10% in acetic acid and adjusted to pH 2.5 with pyridine. After acid cleavage, the mixture was thoroughly dialyzed in Spectrapor Type 3 dialysis tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.) (retains mol wt 3,500) against distilled water and lyophilized.

The lyophilized, succinylated, acid-cleaved light chains were subjected to 15 cycles of automated Edman degradation in a Beckman 890B sequencer (Beckman Instruments, Inc., Fullerton, Calif.) using an improved dimethylallylamine program (111374). The resulting phenylthiohydantoin (PTH) amino acids were identified by gas chromatography (11) and thin-layer chromatography (12). Quantitation of PTH amino acids was done by integration of gas chromatographic tracings and by back hydrolysis with HCl (13) of the PTH amino acids, followed by amino acid analysis with a Durrum D500 analyzer (Durrum Instrument Corp., Palo Alto, Calif.). Norleucine was used as an internal standard in the quantitation procedure. Because the substitutions were a serine for an alanine and a leucine for a glutamine, no single method of quantitation was ideal. However, internally consistent numbers were obtained by using integration of gas chromatographic tracings to estimate serine and alanine, and amino acid analysis after back hydrolysis to measure leucine and glutamine. Serological analyses were performed using radiobinding and inhibition of binding assays as previously described (14).

Results

The sequence analysis of the constant region of the light chain from homogeneous anti-Group C streptococcal antibody 4539 revealed two substitutions, at positions 121 and 124, not previously observed for rabbit b4 light chains. Fig. 1 shows the C**L**-region sequence from position 110–145 for 4539 Ab and for the prototype b4 L chain of 4135 Ab (15). In 4539 serine is found in place of the usual alanine at position 121 and leucine in place of glutamine at position 124. The prototype sequence has been reported for six different homogeneous antibodies and for b4 pools from several different sources (references 4, 16, and 17; and A. L. Thunberg, unpublished data). The partial V**L**-region sequence from 4539 Ab (Fig. 1) shows no unusual features in comparison to 4135 Ab. All V**L**-region variations found between 4539 Ab and 4135 Ab have been observed in other b4 antibodies.

To determine whether the b4**ar** sequence was present in detectable levels in normal IgG from 4539 a pool of light chains was isolated from a bleeding obtained before the third immunization series of this rabbit. This bleeding had no detectable level of antibody to streptococcal antigens. In this pool approximately 32% had the variant sequence, and the remainder had the prototype sequence (Table I). The pattern of inheritance of b4**ar** was ascertained by sequence analysis of L chains obtained from members of the pedigreed family of 4539. As shown in Fig. 2, the ability to express this variant could be traced back four generations to rabbit 2346, which was purchased from a commercial rabbitry. The approximate percentages of b4**ar** and prototype light chains present in serum from rabbits positive for b4**ar** are shown in Table I.

The pedigree depicted in Fig. 2 indicates the members of the 4539 extended family that are positive for the variant trait. A total of 14 related rabbits were tested by L-chain sequence analysis and 7 rabbits displayed this trait. The concentrations of b4**ar** in the related rabbits ranged from approximately 10–32%.
JOHN A. SOGN AND THOMAS J. KINDT

FIG. 1. Comparison of L-chain sequences of antistreptococcal antibodies 4135 (15) and 4539 in the regions 109-145, 0-30, and 62-89.

TABLE I

Level of b4 var in Rabbit Sera

| Rabbit | b4 var* |
|--------|---------|
|        | %       |
| 2246   | 31      |
| 2711   | 20      |
| 3005   | 30      |
| 3553   | 10      |
| 4539#  | 32      |
| 4137   | 28      |
| 4782   | 27      |

* Limit of detection approximately 2%, accuracy ±5%.
# Pooled L chains from a bleeding between immunization periods.

It appears from these data that b4 var is inherited as an autosomal codominant trait. No rabbit putatively homozygous for b4 var was found in this study, but none would be expected on the basis of the crosses performed.

The serologically detected b4 allotypic determinant of 4539 Ab was compared to that of pooled b4 IgG and to that of a homogeneous antibody with the prototype sequence. No differences between L chains with the variant and those with the prototype sequence in their constant regions could be detected by these
serologic tests. Radiolabeled 4539 Ab was bound strongly and specifically to insolubilized anti-b4 serum (14), but did not bind to insolubilized anti-b5, anti-b6, or anti-b9 serum. This binding could be completely inhibited by unlabeled 4539 Ab or pool b4 IgG, or by 4135 Ab, which has the prototype sequence (15). These three preparations were equally effective as inhibitors over the concentration range tested. Binding of radiolabeled pooled b4 IgG to solidified anti-b4 serum could similarly be inhibited completely with identical efficiencies by unlabeled 4539 Ab, 4135 Ab, and pooled b4 IgG.

Discussion

Amino acid sequence analysis of the b4 L chain from homogeneous antistreptococcal 4539 Ab revealed that two C-region positions were occupied by residues not previously observed at these positions. As compared to six b4 L chains previously sequenced through this region (4, 16, 17), the substitutions were serine in place of alanine at position 121 and leucine in place of glutamine at position 124. Quantitative analysis showed that these variant residues occurred in 32% of the L chains from pooled 4539 IgG, while the remaining 68% had the prototype sequence. A family study established that the substitutions represent the product of a stable C_e gene or genes. By contrast, the V region of this L chain exhibited no unusual features.

By the methods used, the limit of detection was approximately 2% b4"^" and the accuracy of quantitation ±5%. The L chains from IgG samples studied always contained less than 35% of the b4"^" sequence, and in the case of rabbit 3553 the level was only 10% (Table I). It can be seen that all values, except those for 3553 (10%) and 2711 (20%), are close to 30%. No significance can yet be attached to this finding. While it was not difficult to detect and quantitate this polymorphism these relatively low amounts might well have been overlooked had the differences in question not first been observed in a homogeneous antibody.
Comparison of prototype Cα sequences for b4, b4™, and b9 in the region 109-130. Brackets denote a deletion and a line indicates sequence identity compared to b4.

The serine residue found in b4™ at position 121 is also observed at position 121 in b9 L chains (Fig. 3). Thus, one of the many apparent distinctions between b4 and b9 is blurred by this finding. The similarity between b4™ and b9 is not reflected in any cross-reactivity between their allotypic specificities. In addition, all attempts to date to differentiate b4™ serologically from b4 have been unsuccessful. Thus there is no apparent relationship between the residues at positions 121 and 124 and group b allotypic determinants.

The present study has concentrated on a single pedigreed rabbit family, and consequently our results provide no information concerning the frequency of the b4™ gene in rabbit populations. Only two unrelated b4 rabbits have been examined for b4™. A rabbit from the same breeder that supplied rabbit 2346 was positive for b4™ while a rabbit from a different breeder was negative.

The group b allotypes were discovered by Oudin (18), and localized to the κ L chain by Stemke (19). While the exact location of the group b determinant on the κ L chain is unknown, the available evidence suggests strongly that it is confined to the C region. Sequence studies of Cα regions have demonstrated extensive differences among L chains of different b allotypes (2). In addition, large variations in N-terminal sequence have no effect on group b serology (20). V-region sequence differences are found among L chains of different group b allotypes (21), but because none of the allotype-associated V-region variants is present in all chains with a given allotype it is most likely that these differences reflect linkage of Cκ genes to Vκ genes. Variation in b4 C-region sequence has previously been reported for position 174 (1, 2) but no genetic data concerning this variation are available.

Although this retrospective family study of the inheritance of b4™ has not distinguished whether the b4™ gene is allelic or pseudoallelic with respect to the b4 gene, prospective breeding experiments will easily answer this question and should place constraints on models of Cκ genes. For example, if crosses of rabbits positive for b4™ never result in offspring expressing the b4™ sequence on all κ-chains, b4™ must be considered a pseudoallele of normal b4. Precedents for pseudoalleles among L-chain genes include Oz, Kern, and other markers for human λ-chains (22) and the allotypes c7 and c21 from rabbit λ-chains (23). That multiple Cκ genes exist is suggested by the presence of group b allotypes on both κ-chain subtypes κs and κb (24, 25).

The existence of multiple pseudoalleles encoding the allotype b4 could explain the unequal synthesis of b4 and b4™ L chains (Table I). Similar inequalities are observed for many allotype systems (26), including the group b allotypes (27), in putatively heterozygous rabbits. Dubiski (28) has called attention to the fact that the inequality usually occurs in a predictable direction, which he calls a “pecking order.” IgG of allotype b4, for example, usually represents 80% of
circulating IgG in b4b9 rabbits (27). Similarly predictable inequalities are seen for L-chain types (κ and λ) and for group a and a-negative H chains (26).

Explanations for unequal synthesis of Cκ allotypes have usually invoked selection by antigen for a specific set of V regions preferentially associated with constant regions of one allotype. If there are multiple b4 (Cκ) genes then gene dosage effects may be postulated as an alternative explanation for the phenomenon of unequal synthesis. That is, the observed quantity of b4var L chains may reflect the percentage of b4var genes in a large b4-gene pool. If this were the case, one might expect some of the offspring from a cross between two rabbits positive for b4var to express levels of b4var near the sum of the levels expressed by the two parents.

Any model proposed for genes involved in the synthesis of immunoglobulins must take into account recent data on the nonallelic behavior of rabbit allotypes of groups a and b. Strosberg et al. (29) have described a single rabbit which expressed, on immunization with Micrococcus lysodeikticus, immunoglobulin with three group a allotypes and three group b allotypes. This observation has been extended by Mudgett et al. (30), who demonstrated low levels of allotypes not detected by qualitative typing procedures or anticipated from breeding data in sera from many rabbits. These findings suggest that the allelic behavior of the rabbit group a and group b allotypes may be established by allelic regulator genes, with each individual possessing structural genes for all of the allotypes in each group. This suggestion is certainly compatible with the existence of either single or multiple copies of the genes encoding each of the group b allotypes.

The present observation of an inherited variant within the genes encoding the allotype b4 provides a new genetic marker with which to probe the number and nature of the genes encoding Cκ regions. The availability of rabbit breeding pairs with the b4var marker and the relative ease with which quantitative data can be obtained should make it possible to determine the number of b4 genes. Furthermore, studies on the idiotypes and Vκ subgroups of different b4var L chains can provide new information about VL-CL interaction.

Summary

Amino acid sequence analysis of a b4 light chain from a rabbit homogeneous antistreptococcal antibody revealed the presence of two amino acid substitutions in the constant region not previously reported for these positions. These interchanges, consisting of serine for alanine at position 121 and leucine for glutamine at position 124, were also present in about 30% of the pooled b4 light chains isolated from pooled IgG from the rabbit (4539) that produced the homogeneous antibody. In addition, these interchanges (b4var) were found, always at the same levels, in varying percentages in nonimmune or early immune bleedings from related rabbits in this pedigreed family and could be traced for five generations. The inheritance pattern of b4var was consistent with autosomal codominant inheritance.

The expert technical assistance of Ms. Donna Atherton is gratefully acknowledged.

Received for publication 1 March 1976.
References

1. Kindt, T. J., and L. E. Mole. 1974. Immunoglobulin structure and rabbit allotypes. In Progress in Immunology, II. L. Brent and J. Holborow, editors. North-Holland American-Elsevier, Amsterdam, The Netherlands. 13.

2. Hood, L., J. H. Campbell, and S. C. R. Elgin. 1975. The organization, expression and evolution of antibody genes and other multigene families. Annu. Rev. Genet. 9:305.

3. Strosberg, A. D., K. J. Fraser, M. N. Margolies, and E. Haber. 1972. Amino acid sequence of rabbit pneumococcal antibody. I. Light-chain cysteine-containing peptides. Biochemistry. 11:4978.

4. Appella, E., O. A. Roholt, A. Chersi, G. Rodzinski, and D. Pressman. 1973. Amino acid sequence of the light chain derived from a rabbit anti-p-azobenzoate antibody of restricted heterogeneity. Biochem. Biophys. Res. Commun. 53:1122.

5. Krause, R. M. 1970. The search for antibodies with molecular uniformity. Adv. Immunol. 12:1.

6. Greenblatt, J. J., D. Bernstin, V. A. Bokisch, T. J. Kindt, and R. M. Krause. 1973. Recovery of large amounts of antibody from immunized rabbits by multiple nonsurgical exchange transfusions. J. Immunol. 110:862.

7. Klapper, D. G., and T. J. Kindt. 1974. Idiotypic cross-reactions among anti-streptococcal antibodies in an inbred rabbit population. Scand. J. Immunol. 3:483.

8. Thunberg, A. L., and T. J. Kindt. 1976. Amino acid sequence of rabbit light chains: variable region of a light chain from a homogeneous immunoglobulin raised by streptococcal immunization. Biochemistry. In press.

9. Fraser, K. J., R. Poulsen, and E. Haber. 1972. Specific cleavage between variable and constant domains of rabbit antibody light chains by dilute acid hydrolysis. Biochemistry. In press.

10. Fleischman, J. B., R. R. Porter, and E. M. Press. 1963. The arrangement of the peptide chains of γ-globulins. Biochem. J. 88:220.

11. Pisano, J. J., and T. J. Bronzert. 1969. Analysis of amino acid phenylthiohydantoins by gas chromatography. J. Biol. Chem. 244:5597.

12. Summers, M. R., G. D. Smithers, and S. Oroszlan. 1973. Thin-layer chromatography of sub-nanomole amounts of phenylthiohydantoin (PTH) amino acids on polyamide sheets. Anal. Biochem. 53:624.

13. Van Orden, H. O., and F. H. Carpenter. 1964. Hydrolysis of amino acids. Biochem. Biophys. Res. Commun. 14:399.

14. Gottlieb, A. B., R. K. Seide, and T. J. Kindt. 1975. Quantitative determination of allotypic and idiotypic markers using antisera coupled to N-hydroxysuccinimide-activated Sepharose. J. Immunol. 114:51.

15. Chen, K. C. S., T. J. Kindt, and R. M. Krause. 1974. Amino acid sequence of an allotype b4 light chain from a rabbit antibody to streptococcal carbohydrate. Proc. Natl. Acad. Sci. U. S. A. 71:1995.

16. Jaton, J. C. 1974. Amino acid sequence of the N-terminal 139 residues of a light chain derived from a homogeneous rabbit antibody. Biochem. J. 141:1.

17. Jaton, J. C. 1974. Comparison of the amino acid sequences of the variable regions of light chains from two homogeneous rabbit anti-pneumococcal antibodies. Biochem. J. 141:15.

18. Oudin, J. 1960. Allotypy of rabbit serum proteins. I. Immunochemical analysis leading to the individualization of seven main allotypes. J. Exp. Med. 112:107.

19. Stemke, G. W. 1964. Allotypic specificities of A- and B-chains of rabbit γ-globulin. Science (Wash. D. C.). 145:403.

20. Kindt, T. J., R. K. Seide, H. Lackland, and A. L. Thunberg. 1972. Serologic identity of the b4 allotypic determinants present on homogeneous rabbit light chains with
different N-terminal amino acid sequences. *J. Immunol.* 109:735.

21. Thunberg, A. L., H. Lackland, and T. J. Kindt. 1973. Sequence variations in b9 light chains as potential genetic markers. *J. Immunol.* 111:1755.

22. Fett, J. W., and H. F. Deutsch. 1976. The variability of human λ chain constant regions and some relationships to V-region sequences. *Immunochemistry.* 13:149.

23. Gilman-Sachs, A., R. G. Mage, G. E. Young, C. Alexander, and S. Dray. 1969. Identification and genetic control of two rabbit immunoglobulin allotypes at a second light chain locus, the C locus. *J. Immunol.* 103:1159.

24. Rejnek, J., E. Appella, R. G. Mage, and R. A. Reisfeld. 1969. Subtypes of rabbit κ light polypeptide chains associated with the b locus. *Biochemistry.* 8:2712.

25. DeVries, G. M., M. Lanckman, and R. Hamers. 1969. Separation of two types of light polypeptide chains from rabbit γ-globulins. *Eur. J. Biochem.* 11:370.

26. Kindt, T. J. 1975. Rabbit immunoglobulin allotypes: structure, immunology and genetics. *Adv. Immunol.* 21:35.

27. Kindt, T. J. 1974. Allelic selection of light chain allotypes by antibodies raised in heterozygous rabbits. *J. Immunol.* 112:601.

28. Dubiski, S. 1972. Genetics and regulation of immunoglobulin allotypes. *Med. Clin. North Am.* 56:557.

29. Strosberg, A. D., C. Hamers-Casterman, W. Van der Loo, and R. Hamers. 1974. A rabbit with the allotypic phenotype ala2a3b4b5b6. *J. Immunol.* 113:1313.

30. Mudgett, M., B. A. Fraser, and T. J. Kindt. 1975. Nonallelic behavior of rabbit variable-region allotypes. *J. Exp. Med.* 141:1448.