Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4883–4886, July 1986
Immunology

Mutation affecting the expression of immunoglobulin variable regions in the rabbit

(allotype/gene expression)

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Communicated by Niels K. Jerne, March 5, 1986

ABSTRACT **We have found a variant of the allotype allele *a2* in the rabbit, which presumably arose by mutation, that segregates as expected for an allele at the *a* locus. This allele is called "*ali*" and the corresponding rabbit strain is called "Alicia." In heterozygous animals (*ali/a1* and *ali/a3*) the concentration of a2 molecules is lower by a factor of 1000 than in standard *a2/a2* homozygotes. In homozygous *ali/ali* individuals the a2 concentration varies with age—i.e., very low in young rabbits and higher in older ones—but it never reaches normal levels. The low level of a2 is compensated by increased amounts of a-negative molecules. Southern blot analysis did not reveal any gross changes in the intron between J_H and C_μ (joining region of immunoglobulin heavy chain and constant region of immunoglobulin μ chain) or in the number of V_H gene segments encoding *a* locus specificities. We suggest that the ali phenotype is due to a mutation in a control element.**

The immunoglobulin (Ig) molecules are made up of heavy (H) and light (L) polypeptide chains. In a few species, the amino acid sequences of these chains and their DNA sequences have been determined (1–3). H chains can be divided into a variable (V_H) region, which contributes to the antibody-combining site, and a constant (C_H) region, which determines the class of the Ig. The H chains of the rabbit are encoded by a cluster of closely linked genetic loci: *a–ms–d–e–f–g*, with a few alleles being known at each locus (2, 4). At the DNA level, the order of gene segments appears to be V_H–J–C_μ–C_γ–C_ε–C_α (where J = joining) (5). Allotypic determinants of the *a* locus are present in V_H regions of all Ig classes (2). Some 5–30% of the IgG molecules lack *a* locus allotypic determinants and are called a-negative. These molecules express other allotypic specificities, encoded by the *w*, *x*, and *y* loci closely linked to the *a* locus (2, 4), and their N-terminal amino acid sequence differs from that of the a-positive molecules (6).

We describe here a variant, presumably a mutant, in which the expression of a-positive molecules is remarkably altered.

MATERIALS AND METHODS

Animals. Rabbits were bred at the institute and were identical by descent with respect to the H-chain haplotypes.

Igs. Normal serum of allotyped rabbits was run over a protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden) column. After extensive washing the bound material was eluted with 1 M acetic acid, neutralized immediately with Tris buffer, and dialyzed against phosphate-buffered saline (PBS). Standard anti-a2 was purified by affinity chromatography on a2 IgG coupled to CNBr-activated Sepharose 4B according to the procedure suggested by the manufacturer (Pharmacia). Ascites fluid of monoclonal 3.11 anti-a2 was obtained from W. van der Loo and used as such. Commercial anti-rabbit Fc was obtained from Jackson Immunochemical (Avondale, PA) and also used without further purification.

Double Diffusion in Agarose Gel. Undiluted serum and antiserum were allowed to diffuse overnight at +4°C in 1.5% agarose Indubiose A45 (L'Industrie Biologique Française, Gennevillier, France) in PBS containing 2% PEG 6000. The level of sensitivity of this modification of the assay is around 10 μg/ml of antigen (7).

Radioinhibition Assay. Polyvinyl microtiter plates were coated at +4°C with isolated conventional anti-a2 antibodies, diluted ascites fluid containing monoclonal 3.11 anti-a2, or diluted goat anti-Fc. After overnight incubation, plates were washed with PBS, blocked with 1% bovine serum albumin for 1 hr, and washed again with PBS. Finally, serial dilutions of inhibitor in 1% bovine serum albumin and 30,000 cpm of ^{125}I-labeled a2 IgG were added to the wells and the mixtures were incubated overnight at +4°C. Afterwards the plates were washed extensively with PBS containing 0.1% bovine serum albumin; then the wells were cut out by using a hot wire and the bound radioactivity was determined. All tests were done in triplicate. The same a2 IgG was used for labeling and for standard curves. The inhibition curves of a1 and a3 IgG with the anti-Fc reagent were similar to that of the a2 IgG, indicating that the V_H determinants did not interfere with this determination, as expected.

Isolation of Liver DNA. High molecular weight DNA from livers of allotyped rabbits was purified according to ref. 8 with slight modifications: nuclei were prepared, after homogenization and Nonidet P-40 treatment of the tissue, by centrifugation through a 30% sucrose cushion. After lysis of the nuclei with $NaDodSO_4$ and proteinase K digestion, the DNA was isolated by phenol/chloroform extraction.

Southern Blot Analysis. Electrophoresis in 0.8% agarose and transfer of DNA to nitrocellulose filters were done as described (9). λ DNA digested by *Hin*dIII was used as the size standard.

Probing for C_μ. The 1.4-kilobase (kb) *Bst*EII fragment of the cDNA clone pμ3 containing almost the entire coding sequence of the C_μ gene segment (10) was used as a probe. Hybridization was done at 42°C in 50% formamide overnight. After hybridization, the filters were washed three times for 30 min at 42°C in 30 mM NaCl/3 mM sodium citrate, pH 7, containing 0.1% $NaDodSO_4$.

Probing for V_H a2. The 0.5-kb *Bst*EII fragment of pμ3 was used. It contains the 5′ untranslated region, the leader, the entire *a2* V_H, and part of the J region of clone pμ3 (11). Hybridization was done at 65°C. Washing was started with 0.3 M NaCl/30 mM sodium citrate and, after exposure of the filter, the washing stringency was raised by rewashing at lower concentrations of NaCl/sodium citrate. All washings

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Abbreviations: kb, kilobase(s); C_H, constant region of immunoglobulin heavy chain; H, heavy; L, light; V_H, variable region of immunoglobulin heavy chain; J, joining.

were done at 65°C and all washing solutions contained 0.1% NaDodSO$_4$.

RESULTS

Origin of the "Alicia" Strain. A male rabbit (no. 2 in Fig. 1) heterozygous at all Ig loci, except *ms*, was mated to 45 females, who produced 52 litters with 252 offspring. Routine antiallotypic antisera reacted normally with the sera of these offspring in double-diffusion tests in agarose. The markers *a1*, *a2*, and *a3* segregated in a Mendelian fashion. An exception was rabbit 5 (Fig. 1), whose serum failed to precipitate with many anti-a2 antisera, so that this rabbit was at first falsely classified as *a1/a1*. Since a classification as *a1/a1* together with the results at the other Ig loci would have meant that rabbit 5 (Fig. 1) was a recombinant, he received special attention. After further breeding, an individual (no. 8 in Fig. 1) with a peculiar *a2* specificity appeared. We assume that the lack of a normal *a2* allotype in the heterozygous rabbits was the result of a spontaneous mutation, which we call "*ali*," and that rabbit 8 (Fig. 1) was an *ali/ali* homozygote.

Table 1 demonstrates the Mendelian behavior of *ali*. We mated 8 males to 36 females, who produced 49 litters with 291 offspring weaned at the age of 2 months. We have established a strain homozygous for *ali*, and we call this strain "Alicia".

Serological Analysis of a2 Molecules in the ali Mutant. The a2 molecules produced by *ali* homozygotes do not differ serologically from those produced by standard *a2*. IgG isolated from individual homozygous (*ali/ali*) rabbits was tested in a radioinhibition assay for the ability to inhibit the binding of standard a2 IgG to an anti-a2 antibody. As shown in Fig. 2*A*, IgG from homozygous (*ali/ali*) individuals inhibits the a2 anti-a2 reaction completely, a result which indicates that all a2 determinants are present in the ali IgG. Nevertheless, 5- to 6-fold more ali IgG was needed to do so, as compared with the standard a2 IgG. This was confirmed in a similar assay (Fig. 2*B*) using a mouse monoclonal anti-a2 antibody that recognizes the majority of a2-bearing molecules.

The presence of a2 molecules in *ali* heterozygotes can also be demonstrated by the radioinhibition assay. We bled individual rabbits, *ali/a1*, *ali/a3*, and *ali/ali*, and estimated the concentration of the total IgG and of the a2 in radioinhibition assays. Fig. 3 shows results for two *ali/ali* and two *a1/ali* rabbits. The *ali* heterozygotes have normal IgG levels, but only a minute fraction of the molecules bears a2 determinants: 5–10 μg/ml—a concentration just beyond the sensitivity of our double-diffusion test. This concentration was independent of age, in contrast to the behavior of homozygous (*ali/ali*) individuals, who also have normal IgG levels but whose a2 levels rise dramatically with age. Starting with 50 μg of a2 molecules per ml at 2 months of age (the earliest bleed), they had 1.5 mg/ml by 10 months of age, but they never reached the normal a2 level even at the age of 18 months (data not shown). The normal level of IgG in these rabbits is due to compensation by increased amounts of a-negative molecules (A. Gilman-Sachs and A.S.K., unpublished). Several additional rabbits of this type have been tested, including *a3/ali* heterozygotes, and similar results have been obtained (data not shown). In heterozygous *a1/ali* and *a3/ali* the lack of a2 is most likely compensated by the *a1* or *a3* allele, since no elevation of a-negative molecules was found in such rabbits (A. Gilman-Sachs and A.S.K., unpublished).

Analysis of the ali Mutant at the DNA Level. There are at least two obvious ways to account for the effects of the *ali* mutation. The mutation might inactivate an element necessary for the normal expression of the a2-positive V_H gene segments. Alternatively, a considerable number of V_H gene segments encoding a2-positive V_H regions might have been lost by deletion or unequal crossing-over. We attempted to distinguish between these possibilities by Southern blot analysis—i.e., examining the size of restriction fragments hybridizing to labeled probes.

As shown in Fig. 4*A*, no differences in the size of the *Eco*RI fragments hybridizing to a C_μ probe could be detected between the parental *a2* and *ali*. This fragment includes almost the entire major intron between J_H and C_μ (5).

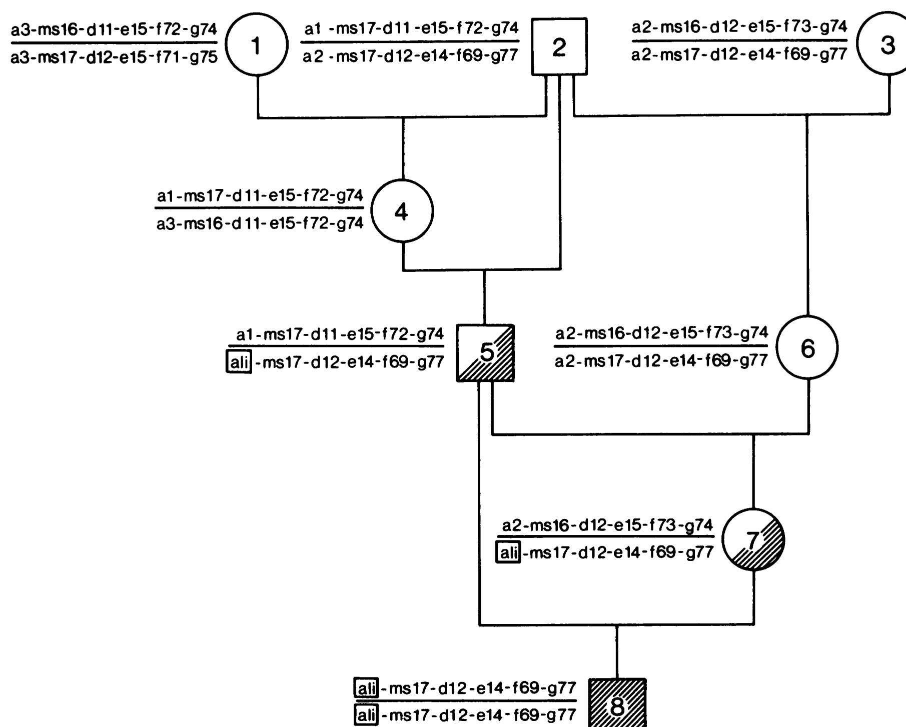

FIG. 1. Pedigree of the family in which the variant *ali* (no. 5) occurred. Rabbit sera were tested for all of the markers indicated, by double diffusion in agarose, as described (7).

Table 1. Distribution of allotypic markers in the progeny of crosses involving the ali mutant

Genotype of the parents	Allotype of the offspring (genotype)									
	ali	*ali/a1*	*ali/a2* *	*ali/a3*	*a1/a2*	*a1/a3*	Total	χ^2	df	*P*
ali/ali × *ali/ali*	22	—	—	—	—	—	22	—	—	—
ali/ali × *a1/a1*	—	18	—	—	—	—	18	—	—	—
ali/ali × *ali/a2*	7 (9)	—	11 (9)	—	—	—	18	0.88	1	0.50–0.75
ali/ali × *ali/a3*	21 (18)	—	—	15 (18)	—	—	36	1.00	1	0.50–0.75
ali/ali × *a3/a3*	—	—	—	16	—	—	16	—	—	—
ali/a1 × *a2/a2*	—	—	8 (7)	—	6 (7)	—	14	0.28	1	0.25–0.50
ali/a1 × *a3/a3*	—	—	—	19 (23)	—	27 (23)	46	1.39	1	0.75–0.90
ali/a1 × *ali/a2*	12 (16.5)	17 (16.5)	19 (16.5)	—	18 (16.5)	—	66	1.26	3	0.25–0.50
ali/a1 × *a2/a3*	—	—	6 (7)	5 (7)	9 (7)	8 (7)	28	1.43	3	0.25–0.50

df, Degrees of freedom. Numbers in parentheses are the expected values used for the χ^2 calculation.
*ali was distinguished from *a2* by its linkage to a different haplotype.

Regulatory elements have been defined in this region in the mouse (12, 13). Four other enzymes (*Bgl* II, *Hin*dIII, *Sac* I, and *Xba* I) have also been used and revealed no difference between *ali* and *a2* (Fig. 5). This sort of analysis would, of course, reveal gross changes only, so the absence of detectable differences does not exclude the first possibility.

The loss of a considerable number of V_H gene segments should be accompanied by a loss of restriction fragments detectable with an *a2* V_H probe. Southern blot analysis of the parental *a2* and *ali* did not show any differences, even when low-stringency washing was used to detect as many V_H gene segments as possible (Fig. 4*B*). The patterns of the parental *a2* and *ali*, though complex, were identical. Of course, this does not exclude the loss of V_H gene segments for which we cannot probe. Nevertheless, this probe detects about 30 bands of various intensities, and we feel that under our conditions of hybridization the probe detects most of the a2-positive V_H gene segments.

DISCUSSION

We have established a variant strain of rabbits that originated from a heterozygous *a1/a2* male. This makes it highly unlikely that the marker *ali* was introduced into the colony from outside but not noticed before. It rather suggests that the ali phenotype is due to a true mutation. The nature of this mutation is very puzzling. We could show that all of the serological determinants usually found in conventional a2 rabbits are also present in ali rabbits. However, the amount of a2 expressed is strongly influenced by the mutation.

At the DNA level no differences between ali and parental a2 could be found. Neither the size of restriction fragments containing the C_μ gene segment nor the pattern and the number of restriction fragments hybridizing to an *a2* V_H probe showed any alteration. On balance, we favor the hypothesis that the *ali* mutation affects an element responsible for optimal a2 expression.

Several years ago, we described the *bas* mutation, which results in the loss of expression of the nominal κ allotypes representing up to 90% of the L-chain pool (7). This loss was compensated in homozygous *bas/bas* rabbits by a huge increase of a "new" L-chain isotype (κ_2) and of λ chains (7, 14). This compensation facilitated the characterization of isotypes—experimentally difficult under normal conditions. The bas phenotype turned out to be due to a mutation at a splice acceptor site (15).

We think that, in an analogous fashion, further analysis of Ig of *ali* rabbits will throw new light on the a-negative markers. There are obvious advantages of studying the control of Ig expression *in vivo* rather than in cell lines.

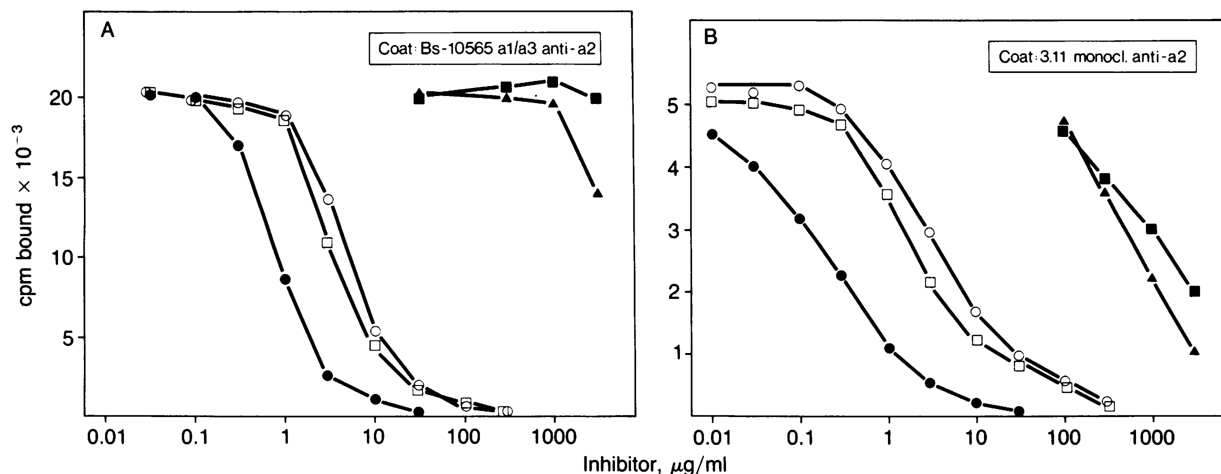

FIG. 2. Inhibition of binding of radiolabeled a2 IgG to conventional and monoclonal anti-a2 by IgG from individual *a1*, *a2*, *a3*, and *ali* homozygous rabbits: IgG from serum of individual homozygous *a1* (▲), *a2* (homologous to the labeled IgG) (●), *a3* (■), and *ali* (○ and □).

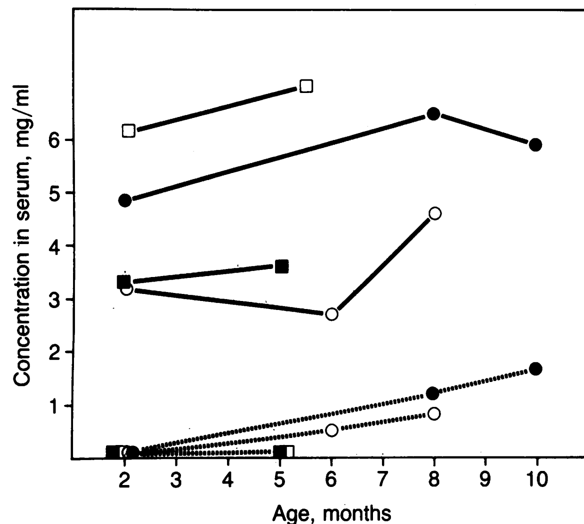

FIG. 3. Quantification of IgG and a2 in serum of individual *ali/ali* or *a1/ali* rabbits at different ages. Concentrations were determined by using commercial goat anti-rabbit Fc and the anti-a2 of Fig. 2*A*. The same a2 IgG as in Fig. 2 was used for labeling and for the standard curve. Two individual *ali/ali* (○ and ●) and two individual *a1/ali* (□ and ■) are shown. The solid line (—) represents the concentration of total IgG and the broken line (---) represents the concentration of a2-bearing molecules in serum of the individual rabbits, respectively. The amount of a2 in the serum of the two *a1/ali* rabbits was between 5 and 7.5 μg/ml.

Finally, since V_H regions, which normally dominate an immune response, are almost absent in young *ali* homozygotes, the Alicia strain of rabbits represents a unique model for studying the establishment of the immunological repertoire in the context of an idiotype network (16).

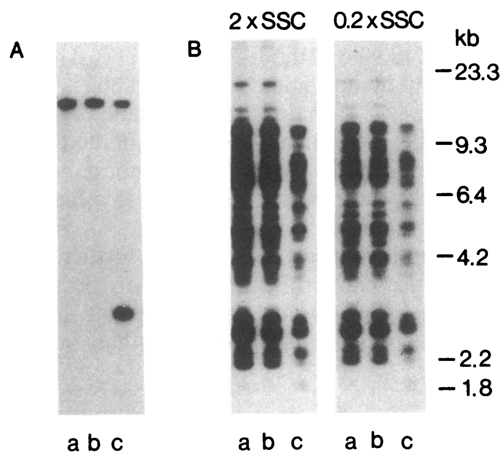

FIG. 4. Southern blot analysis of liver DNA of individual homozygous *a1*, *a2*, and *ali* rabbits. Lanes a, *a2/a2*: lanes b, *ali/ali*; lanes c, *a1/a1*. SSC, standard saline citrate (0.15 M NaCl/15 mM sodium citrate, pH 7). (*A*) *Eco*RI-digested DNA probed with the 1.4-kb *Bst*EII fragment of the cDNA clone pμ3 containing almost the entire C_μ gene segment (10). It is not clear why there is an additional band at 2.8 kb in the *a1/a1* rabbit. (*B*) *Eco*RI-digested DNA probed for V_H *a2* gene segments at different stringencies with the 0.5-kb *Bst*EII fragment of pμ3 (11).

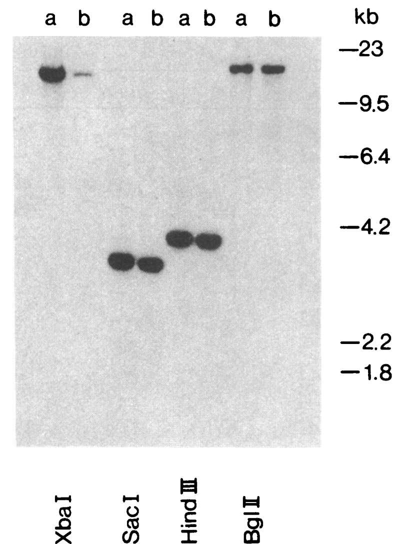

FIG. 5. Southern blot analysis of liver DNA of homozygous *a2* and *ali*. DNA assay conditions and the probes were identical to those used in Fig. 4*A* except that different enzymes were used for digestions, as indicated. Lanes a, *a2/a2*; lanes b, *ali/ali*.

We thank Drs. R. G. Mage (National Institutes of Health) for the gift of rabbit 2 and for the probes and W. van der Loo (Free University of Brussels) for the monoclonal anti-a2 antibody. Our thanks also go to Drs. W. C. Hanly and A. Gilman-Sachs for performing some serological tests. We are indebted to B. Johansson, D. Meier, and W. Hänggi for excellent technical help. We are especially grateful to Drs. C. M. Steinberg and L. Du Pasquier for useful discussion and criticism of the manuscript. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland.

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