

ISOLATION AND CHARACTERIZATION OF IgG MOLECULES EXPRESSING LATENT GROUP b ALLOTYPES FROM PEDIGREED b⁴b⁴ RABBITS*

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Genetic studies have indicated that immunoglobulin allotypes are inherited as codominant Mendelian alleles and that their synthesis is controlled by genes at three unlinked loci (1). However, recent evidence has challenged the notion that immunoglobulin allotypes are encoded by allelic structural genes, and has led to the suggestion that individuals may possess complex groups of structural genes, the expression of which is governed by specific regulatory genes (2-4). The generality of nonallelic behavior of allotypes was initially suggested by the frequent occurrence of low levels of latent group a allotypes in rabbit sera (5).

In our laboratory and elsewhere, immunoglobulin and antibody molecules bearing latent group a and group d allotypes have been thoroughly characterized using serological, and in some instances, structural techniques (4-9).¹ Nevertheless, questions have been raised concerning the validity of latent allotype observations. For example, it has been contended that the heterogeneity, multiplicity, and complexity of the heavy chain variable region structures on which the group a allotypic determinants reside make unambiguous identification of latent a allotypes an extremely difficult task (10). Others have suggested that latent components may be the products of lymphoid cells from mother or siblings which enter the fetus during pregnancy. These issues might be resolved if serologically distinct, structurally defined latent markers were expressed in rabbits of defined pedigree. Recent serologic (11) and structural studies (12-15) have indicated that the group b allotypes of rabbit light chains are ideally suited for this purpose.

The present study presents evidence for the occurrence of latent b allotypes in sera from pedigree rabbits. Sera from 37 homozygous b⁴b⁴ rabbits were tested by a sensitive radioimmunoassay (RIA),² and 5 were found to contain significant concentrations of either latent b5 or latent b9 allotypes. Furthermore, in two instances, the level of latent b9 allotypes was sufficiently high to permit

* Latent allotypes are allotypes not predicted by breeding data nor detected by normal qualitative typing procedures. The term nominal allotype is used for those determined by usual typing procedures consistent with breeding data.

¹ M. Mudgett-Hunter, M. L. Yarmush, B. A. Fraser, and T. J. Kindt. 1978. Rabbit latent group a allotypes: characterization and relationship to nominal group a allotypic specificities. Manuscript submitted for publication.

² Abbreviations used in this paper: BSA, bovine serum albumin; H, heavy; HAS, N-hydroxy-succinimide-activated Sepharose 4B; L, light; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

isolation and detailed serologic characterization of the immunoglobulin population bearing this allotype.

Materials and Methods

General. Techniques for preparation of streptococcal vaccines and immunization of rabbits have been previously described (16). Rabbits used in this study (allotypes b⁴b⁴) were selectively bred at The Rockefeller University to give high antibody responses of restricted heterogeneity after streptococcal immunization. Anti-allotype sera were prepared according to the principles of Oudin (17) with previously described modifications (1). The presence of anti-allotype antibodies was determined by capillary precipitin test, and the specificity of the antisera was checked by RIA (18). Pools of nonimmune IgG from rabbits homozygous for a and b allotypes were purified by DEAE-cellulose chromatography using 0.02 M potassium phosphate buffer as eluant (pH 7.0) for b₄ IgG, 0.03 M phosphate buffer for b₅ and b₆ IgG, and 0.04 M phosphate buffer for b₉ IgG. IgG from at least three rabbits was included in each pool. Each pool was checked for the presence of latent allotype, and only those pools that tested negative by RIA were used. IgG concentrations in rabbit sera were determined by radial immunodiffusion using goat anti-rabbit Fc-γ sera in 1% agarose, 0.05 M phosphate-buffered saline (PBS), pH 7.0 (19). The extent of radial diffusion of each sample was measured after 36 h, and concentrations were assigned by reference to IgG standards. The isolation of an homogeneous anti-streptococcal antibody of allotype a₃/b₉ from the transfusion plasma of rabbit 4153 has been previously described (20).

Immunoabsorbent Columns. Columns used for removal of cross-reactive anti-allotype antibodies were prepared by coupling 50 mg of IgG of known allotype to 25 ml of Sepharose 4B by the cyanogen bromide method of Cuatrecasas (21), and then mixing equal quantities of three IgG immunoabsorbents containing the group b allotypes that were expected to be nonreactive with the anti-allotype serum under investigation. All immunoabsorbent columns used in this study were washed with 3 M NH₄SCN in PBS, pH 7.0, before use.

Radioiodination. In most instances, purified antibody and IgG preparations were radiolabeled using iodine monochloride (22). Occasionally samples were radioiodinated using immobilized lactoperoxidase (23).

RIA. Insolubilized antisera were prepared by coupling the sera to *N*-hydroxysuccinimide-activated Sepharose (HAS), as described by Gottlieb et al. (18). All antisera were heat-activated, filtered, and passed through the appropriate adsorption column before immobilization. Nominal allotypes were determined by RIA as previously described (18). Binding curves were constructed using 0.25 μg of radiolabeled antigen and several dilutions of HAS-anti-allotype in a total volume of 200 μl. Inhibition analyses were performed using concentrations of antisera that bound 50% of the radiolabeled antigen. Latent b allotypes were quantitated as described for the latent a allotypes (5) with some modification. Inhibitor (100 μl) and 0.25 μg of radiolabeled IgG antigen (50 μl) were added to Beckman microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.). Antigen was diluted in PBS containing 0.04 M dithiothreitol and carrier protein. Carrier proteins included 1% bovine serum albumin (BSA) and 10% nonimmune rabbit serum which contained a 50- to 100-fold excess of all group a and b allotypes except the one under investigation. The contents were mixed thoroughly and allowed to stand at room temperature for 30 min. Insolubilized anti-allotype serum (50 μl), at a dilution which bound 50% of the radiolabeled antigen, was then added and the contents were again thoroughly mixed. The tubes were agitated for 2 h at room temperature, centrifuged, and 100 μl of supernatant fluid was transferred to a second tube. Supernate and precipitate fractions were counted to determine the percentage of label bound to the HAS. Percent binding was calculated as follows:

$$\% \text{ binding} = \frac{P - S}{P + S - 2B}$$

where P is cpm of the precipitate, S is cpm of the supernatant fluid, and B is background cpm. Percent inhibition was calculated as described by Kindt et al. (24). Inhibition values <10% were considered negative. All inhibitor test sera were heat-inactivated and filtered before assay.

Heavy (H) and Light (L) Chain Separation. IgG was separated into H and L chains by mild reduction and alkylation followed by chromatography on Sephadex G-75 in 10% acetic acid,

according to the principles of Fleischman et al. (25). H chains were stored in distilled water; L chains were stored in PBS, pH 7.0.

Isolation of Anti-Allotype Antibodies. Anti-b9 antibodies were isolated by applying anti-b9 sera to an immunoabsorbent column containing 50 mg of a3/b9 IgG covalently attached to 25 ml of Sepharose 4B. After extensive washing with PBS, the bound fraction was eluted with 3 M NH_4SCN , dialyzed against PBS, and then passed through an adsorption column containing all allotypic specificities except b9. A total of 18 mg of anti-b9 antibody was isolated from 120 ml of anti-b9 serum.

Isolation of Latent b9 Molecules. A specific anti-b9 allotype immunoabsorbent was prepared by coupling 18 mg of purified anti-b9 antibody to 10 ml of Sepharose 4B. The coupled antibody was divided into four acid-washed, siliconized columns, and each column was conditioned with 10% normal rabbit serum containing all allotypic specificities except b9. In a typical experiment, IgG from a serum containing latent b9 was radiolabeled and passed through an anti-b9 column. The column was washed extensively with PBS and the bound fraction was eluted with 3 M NH_4SCN in PBS, pH 7.0. BSA was added to the eluate to a concentration of 1%, and the resulting mixture was dialyzed against PBS. Each sample of isolated, latent b9 antibodies was then passed through a fresh column containing 2 ml of Sepharose 4B to which 5 mg of anti-b4 IgG was coupled. The effluent was concentrated and tested for binding to various anti-allotype sera. The purpose of the second column was to remove rheumatoid factors, denatured protein, and residual b4 antibodies which may have nonspecifically bound to the first immunoabsorbent column.

Results

Listed in Table I are the genotypes of the rabbits in which anti-allotype sera were prepared, the immunogens used, and the antigens employed in the inhibition of binding assays. Each anti-allotype serum was prepared in a rabbit heterozygous at the b locus and passed through the appropriate preadsorption column before use. Antisera were immobilized on HAS and tested for cross-reactions with the other three b allotypes. Each anti-allotype serum bound only the radiolabeled antigen to which it was directed and showed no binding to the three other b allotypes. Standard inhibition curves for each allotype were generated by using increasing concentrations of the appropriate unlabeled allotype pools as inhibitors. Only homologous pools inhibited the binding of the radiolabeled pools to their respective HAS-anti-allotype sera, whereas none of the heterologous pools gave significant inhibition values in the homologous systems. Illustrated in Fig. 1 are standard inhibition curves for b5 and b9 allotypic reactions.

Screening for Latent Allotypes. A search for latent b allotypes was initiated using the specific anti-allotype sera described above. Nonimmune sera from 37 pedigreed homozygous b^4b^4 rabbits were tested (Fig. 2). The presence of latent allotypes was determined by the ability of test sera to inhibit the binding of radiolabeled IgG of known allotype to its anti-allotype serum. Reported in Table II are inhibition values obtained using undiluted sera as inhibitor. Significant levels of latent b allotypes were detected in 5 of the 37 sera tested; rabbits 2896 and 2710 contained latent b9, rabbits 3007 and 2667 contained latent b5, and rabbit 2363 contained both latent b5 and latent b9. Latent b6 was not detected in any of the sera tested. Approximate concentrations of latent allotypes listed in Table II were assigned from standard inhibition curves (Fig. 1).

IgG was isolated from the sera of rabbits 2896, 2710, and 2363, concentrated threefold, and used as inhibitor in b9 binding reactions. Concentrated IgG preparations from 2896 and 2710 inhibited >90% of the binding, whereas only 60% inhibition was achieved with 2363 IgG (Table III).

TABLE I
Reagents Used in This Study

Specificity	Rabbit*	Genotype	Allotype of immunogen	Allotype of radiolabeled IgG antigen†
Anti-b4	4766	a ² a ³ /b ⁹ b ⁹	3,4	3,4
Anti-b5	4285	a ² a ³ /b ⁴ b ⁹	3,5	3,5
Anti-b6	4023	a ¹ a ³ /b ⁴ b ⁵	1,3,6	3,6
Anti-b9	4133	a ¹ a ³ /b ⁴ b ⁵	3,9	3,9

* Used to prepare antigen.

† IgG used as antigen in standard RIA.

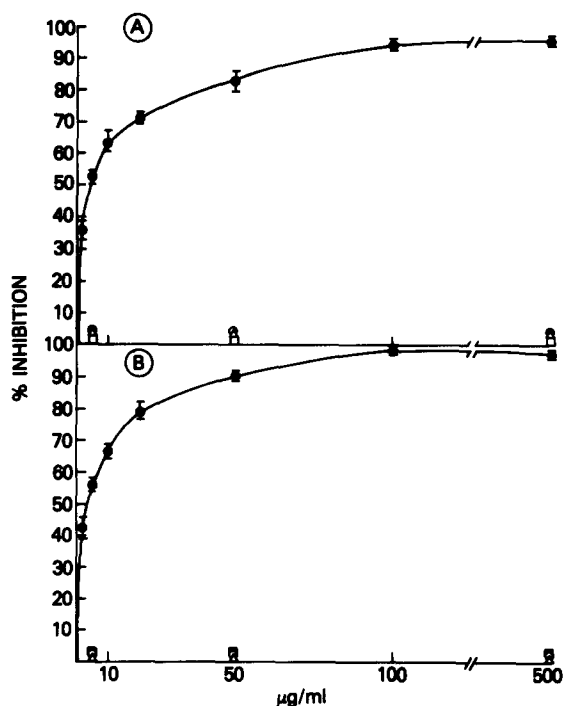


FIG. 1. Inhibition of binding of ¹²⁵I-IgG pools to HAS anti-allotype sera by various IgG pools. (A), inhibition of binding of HAS anti-b5 to ¹²⁵I-a3/b5 IgG by a3/b5 IgG (●), by a3/b4 IgG (Δ), by a3/b6 IgG (○), and by a3/b9 IgG (□). (B), inhibition of binding of HAS anti-b9 to ¹²⁵I-a3b9 IgG by a3/b9 IgG (●), by a3/b4 IgG (Δ), by a3/b6 IgG (○), and by a3/b5 IgG (□).

When homogeneous antibody 4153 was used as radiolabeled antigen, however, the concentrated IgG preparation from 2363 gave nearly 90% inhibition (Table III). The b9 allotypic determinants on L chains of 4153 Ab were previously shown to be serologically deficient with respect to the determinants of b9 pools and two other homogeneous antibodies (6, 26). This result indicates that the latent b9 molecules present in the sera of 2363 also lack one or more of the determinants present in normal b9 pools.

H and L chains were separated from IgG of rabbits 3007 and 2710, and the isolated chains were tested for inhibition in the b5 and b9 assays, respectively. In both cases, a 360-fold molar excess of L chains gave >85% inhibition, whereas

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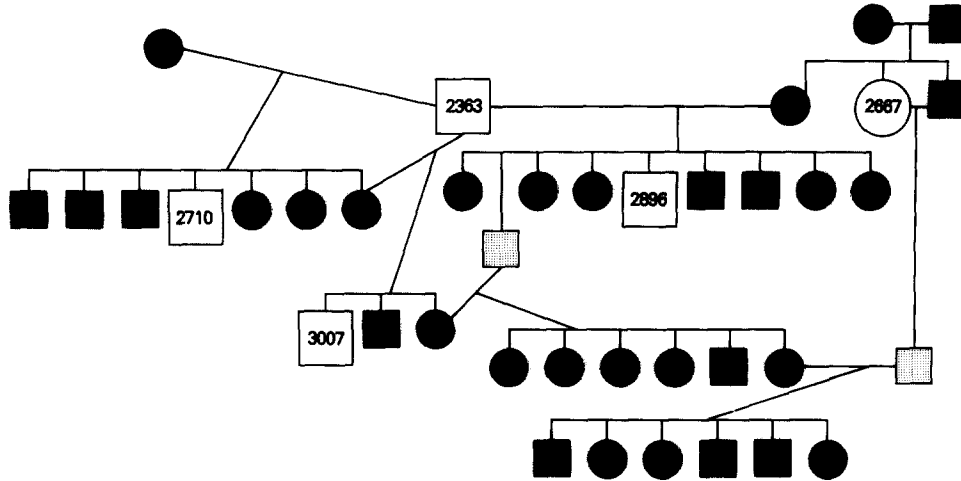


FIG. 2. Pedigree of rabbits bred for homozygosity at b locus. All rabbits were typed b4b4 by RIA (17). Illustrated are rabbits typed positive for latent markers (numbers shown), rabbits typed negative (black figures), and untested rabbits (shaded figures). The presence of latent allotype was determined by the ability of test sera to inhibit the binding of the appropriate radiolabeled IgG pool to its respective HAS anti-allotype serum. Values <10% were considered negative.

TABLE II
Latent Allotype Activity as Measured by RIA

Rabbit	Nominal sero- type	Inhibition*		Concentration of latent allotype†	
		b5	b9	b5	b9
		%		µg/ml	
2896	a1a3/b4b4		82		27.0
2710	a1a3/b4b4		74		16.0
2363	a1a3/b4b4	19	54	1.5	4.5
3007	a1a3/b4b4	50		4.5	
2667	a2a3/b4b4	39		3.0	

* The values listed represent percent inhibition of binding of the appropriate radiolabeled IgG pool to its respective anti-allotype serum.

† These values were assigned using standard inhibition curves illustrated in Fig. 1. IgG concentrations in test sera ranged from 3.3 to 6.8 mg/ml as measured by radial immunodiffusion (19).

an identical molar amount of H chains did not inhibit to a significant degree. The lower inhibition values obtained with isolated L chains versus those obtained with intact molecules are predictably caused by the suboptimal conformation of allotypic determinants on isolated L chains, and by denaturation induced during chain separation.

Immune bleedings from the latent positive rabbits were also examined for the presence of latent b markers. Sequential observations over a period of several weeks revealed marked fluctuations of latent allotype concentrations. Representative examples are shown in Fig. 3.

TABLE III
Latent Allotype Activity in Concentrated IgG Preparations as Measured by RIA

Rabbit	Radiolabeled antigen	Maximum inhibition %
2896	b9 IgG	96
2710	b9 IgG	93
2363	b9 IgG	60
2896	4153 Ab	96
2710	4153 Ab	95
2363	4153 Ab	89

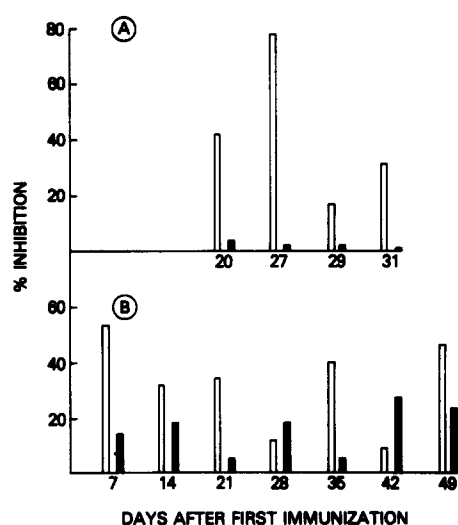


FIG. 3. Latent b5 (dark bars) and b9 (white bars) in anti-streptococcal sera from rabbits 2710(A) and 2363(B). Latent allotype activity is represented as the percent inhibition of binding of the appropriate radiolabeled IgG pool to its respective HAS anti-allotype serum.

Isolation of Latent b9 Molecules. To better characterize the latent components in the test sera, molecules bearing the b9 determinants were specifically isolated from the sera of rabbits 2896 and 2710. Individual IgG preparations were isolated and radiolabeled, and latent b9 antibodies were isolated on anti-b9 immunoabsorbents. As seen in Table IV, in both cases, HAS anti-b9 bound >75% of the purified preparations, whereas the other immobilized anti-allotype sera reacted with <20% of the molecules. Low binding values obtained with anti-b5 and anti-b6 probably represent slight denaturation caused by the elution procedure, whereas binding values obtained with HAS anti-b4 more likely reflect a slight contamination of b4 determinants in the isolated latent b9 preparations. Binding of the purified preparations to several immobilized IgG pools (b4, b5, b6, and b9) was also below 10%, indicating that anti-allotype antibodies were not present in the preparations. In addition, binding of radiolabeled latent b9 molecules to anti-b9 could be inhibited only by b9 IgG; no inhibition was observed with b4, b5, or b6 IgG preparations.

TABLE IV
Serologic Characterization of the Isolated Latent b9 Molecules

Rabbit	Amount eluted*	Anti-b4	Anti-b5	Binding	
				Anti-b6	Anti-b9
	μg			%	
2896	44	17	4	5	80
2710	31	19	8	5	76

* 10 mg of radiolabeled IgG was applied to the columns.

TABLE V
Reciprocal Binding of Latent b9 Molecules from Rabbit 2896

First adsorption with:*	Binding	Second adsorption with:	Binding‡
	%		%
Anti-b4	17	Anti-b9	76
Anti-b9	80	Anti-b4	16

* 1 μg of radiolabeled isolated material was adsorbed twice with either anti-b4 or anti-b9. The supernatant fractions were then tested for binding by the reciprocal addition of the second antiserum.

‡ This value represents the percent of material bound by the addition of the second antiserum multiplied by the percent of unbound material from the first incubations.

To ascertain whether the b4 and b9 determinants reside on separate molecules, sequential binding experiments were conducted with the isolated material from 2896. The isolated preparation (1 μg) was adsorbed twice with either HAS anti-b4 or HAS anti-b9, and the supernate from the second adsorption was tested for binding with the other immobilized antiserum. As shown in Table V, when the isolated preparation was incubated twice with HAS anti-b4, a total of 17% of the molecules was bound. Of the remaining material (83%), 91.5% was bound by HAS anti-b9. Thus, a minimum of 76% of the material reacted only with anti-b9 and not with anti-b4. When adsorptions were carried out in the reverse order, it was found that 16% of the molecules reacted only with anti-b4 and not with anti-b9. These observations indicate that a minimum of 92% of the purified preparation consists of allotypically monoreactive molecules.

The effects of b9 immunoadsorption on the b4 activity in the starting material were also examined. Unlabeled IgG from rabbit 2710 was passed through an anti-b9 column, and the effluent was tested for its ability to inhibit the b4 and b9 allotype binding reactions. Adsorption removed >80% of the latent b9 activity. However, this treatment had no effect on the capacity of the sample to inhibit the binding of a radiolabeled b4 pool to HAS anti-b4.

Discussion

This report documents the occurrence of latent b allotypes in sera from pedigreed rabbits. Serologic analysis of sera from an extended family of homozygous b4 rabbits revealed the presence of latent b allotypes in 5 of the 37

sera tested. Latent b5 and b9 markers were identified; none of the sera tested contained latent b6. N. Francis and W. J. Mandy (personal communication) have also recently found unexpected L chain markers (b4, b5, and b6, but not b9) in preimmune and immune bleedings from rabbits hyperimmunized with ovalbumin.

Since the initial observation of latent allotype expression, efforts have been directed toward proving that these components are identical to those normally expressed, thus eliminating explanations for latent allotype activity based on serologic artifacts. The b4 and b9 allotypes are especially well-suited for this type of study because the polymorphic forms are readily discernible by serologic tests (11). Furthermore, amino acid sequence studies of the b4 and b9 allelic forms have revealed an unusual degree of structural diversity; their respective constant regions differ at $\approx 33\%$ of their positions (13).

However, because cross-reactions among rabbit group b allotypes, notably between b4, b5, and b6, have been reported (10, 27, 28), care must be taken in their determination. One obvious precaution involves the uses of appropriate heterozygotes for serum preparation (11). In order to insure that b locus antisera used in this study were monospecific, antisera were raised in rabbits heterozygous at the b locus, and were then passed through adsorption columns containing an IgG pool with all the group a and b specificities except the one against which the antiserum was directed. All antisera prepared in this fashion were free of cross-reactivity by the criteria of binding and inhibition of binding analyses with allotypically homozygous IgG pools. In addition, rabbit sera containing all the allotypes except the one in question were added to the inhibition assays. Using such measures, it has already been shown that latent group a activity measured by our radioimmunoassay reflects neither nonspecificity nor anti-globulin activity.¹ The results presented here also eliminate these interpretations as explanations for latent b activity. Latent b allotypes were detected in IgG preparations and on isolated light chains, and latent molecules isolated on anti-allotype columns showed little or no binding to normal IgG. Finally, the identification of latent markers in rabbits of defined pedigree precludes the possibility that latent allotypes are derived as a result of permanent chimerism arising from transplacental passage of lymphoid cells during pregnancy.

The values listed in column four of Table II deserved extended comment. In general, inhibition values and not absolute concentrations have been reported in this paper. By comparison of these values with inhibition values obtained using known amounts of homologous inhibitor, concentration values may be obtained as they were for the purposes of this table. It must be emphasized that the extrapolated concentrations are only a convenient way to report latent allotype activity and would have physical significance only if the latent allotypes could be shown by alternative means to be identical to nominal allotypes. Because rabbit group a and b allotypes are each multideterminant antigenic species, mathematical analysis of classical binding or inhibition curves can yield only average apparent association constants. Quantitation by comparison to standard inhibition curves is possible only if each molecule bears all of the determinants recognized by the appropriate anti-allotype serum.

However, this is not the case. Subspecificities of group a allotypes are common and well-characterized (24, 29-31), and subspecificities of group b allotypes, although less common, have been reported (6, 26). In this situation, inhibition values will be dependent on the subspecificity distributions in the labeled antigen, the homologous inhibitor, and the experimental inhibitor, and on the spectrum of subspecificity recognition by the anti-allotype serum. Thus, until independent, complementary, structurally-based quantitation of the molecules bearing the latent allotype is obtained, concentration values for latent allotypes will remain tentative.

It was of particular interest to ascertain whether molecules expressing latent b allotypes comprise a subset of molecules with dual allotypic reactivity. Recent studies have suggested that individual IgG molecules from members of the *Sylvilagus* and *Lepus* genera, may possess determinants belonging to more than one group b allotype (32-35). For example, in one study (34), five different b allotype phenogroups were identified among eight cottontails tested: b(4)', b(6)', b(4,6)', b(4,5,6)', and b(4,6,9)'. All cottontail IgG preparations bound group b anti-allotype sera prepared in domestic rabbits, but these IgG preparations were found to be poor or ineffective inhibitors of binding of domestic rabbit IgG to anti-allotype sera. In studies on rabbit a-locus markers (36), van der Loo et al. have reported that most domestic rabbits of allotype a1 produce a population of molecules which can be detected with a3 anti-a2 sera. Because these molecules were found in significant concentrations in sera of normal hares, the authors suggested that the hare-like molecules expressed in domestic rabbits may be encoded by phylogenetically ancient genes which have remained structural components of the *oryctolagus* genome.

The data reported here, however, indicate that latent b allotype molecules are allotypically monoreactive and serologically identical to nominal allotypes. It is, therefore, unlikely that the latent group b allotypes described here and the latent H chain allotypes described previously are products of unidentified ancient genes or phenogroups. Although the behavior of latent allotypes in serologic analysis parallels that of their nominal counterparts, final confirmation of identity between latent and nominal forms awaits complete structural analysis.

It is obvious that genetic models formulated to account for antibody diversity must be expanded to accommodate these and other similar data. The presence of unexpected immunoglobulin gene products argues that structural genes encoding these molecules must be present in the individual. It has been suggested that the genetic information for every allotype exists on each chromosome as linked genes, and that the allelic behavior of antibody expression is conferred by regulator genes (2). A possible alternative involves the existence of two (or more) closely linked allelic genes for each allotype locus on each chromosome: one of which is activated, encoding nominal allotypes, and the other(s); silent, encoding latent allotypes. Thus, for example, if only two b-locus genes were present on each chromosome, a homozygous individual could express one or two but never three latent allotypes. Studies on the effect of genetic background on latent allotype expression are in progress and may provide some insight into the genetic mechanisms governing allotype expression.

Summary

Latent group b markers were detected in sera, in IgG preparations, and on isolated L chains from rabbits bred for homozygosity at the b locus. Serologic analysis of sera from an extended family of homozygous b4 rabbits revealed the presence of latent b allotypes in 5 of 37 sera tested. Latent b5 and b9 markers were identified; none of the sera tested contained latent b6. In two instances, the level of latent b9 allotypes was sufficiently high to permit isolation and detailed serologic characterization of the immunoglobulin population bearing this allotype. The fact that latent allotypes were detected in pedigreed homozygous rabbits minimizes the possibility that lymphoid cell chimerism is involved in latent allotype expression. Furthermore, characterization of the b9 IgG population indicates that the latent allotypic determinants do not reside on a subset of molecules with dual allotypic reactivity.

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