QUANTITATIVE DISTRIBUTION OF THE GROUP a ALLOTYPES IN NORMAL HETEROZYGOUS SERA

by

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SECTION I

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SECTION II

LITERATURE REVIEW

A. Introduction

The genetically controlled polymorphic forms of rabbit immunoglobulins were first discovered by Oudin (123 - 125) in 1956. Oudin observed that rabbit antibodies were immunogenic when injected into certain other rabbits. Oudin coined the term "allotypy" to describe this phenomenon and he named the intra-species epitopes on the antibodies "allotypes." An important aspect of this discovery was that it provided the first indication of the polymorphic nature of serum proteins among individuals of a single species. These epitopes provided probes for later studies on the genetic control of antibody synthesis.

As data began to accumulate on allotype studies from other investigators a committee on terminology was formed and it was decided that the term allotype would be used as a general definition to describe any protein which exists in polymorphic forms in different individuals of an animal species (29). When more information became available regarding molecular structure (section D) it became apparent that allotypy could be more precisely defined by amino acid substitutions, and that allotypic variation could occur in the absence of antigenic differences.

Early investigations of rabbit allotypy provided important basic information at the genetic level regarding antibody synthesis. In 1962 Dubiski et al. (43) were able to show that Oudin's two groups of allotypes (126) were unlinked. Shortly following was the discovery by Stemke (157) that the alleles

of these two groups are expressed as markers on the heavy and light chains of IgG molecules. Thus it was concluded that the two chains of the antibody molecule were encoded by independently assorting genes. Other early findings included allelic exclusion in cells producing immunoglobulins (129), symmetry of the multi-chained immunoglobulin molecules with respect to their allo-types (30) and the discovery by Todd (165) that V_H allotypes were common among the immunoglobulin classes thus suggesting that a single polypeptide chain could be encoded by more than one gene.

B. Immunization and Detection Procedures

Antiserum directed against rabbit immunoglobulin allotypes is generally prepared by immunization of a second rabbit lacking the allotype of interest. A recipient possessing all other allotypes present in an injected sample will normally respond against only the epitope it does not possess since a rabbit is normally tolerant of epitopes on its own immunoglobulins (126). Prolonged immunization is usually required to elicit anti-allotype antibodies (120).

Allotypic antisera were originally raised by injecting an antigen-antibody precipitate, formed between ovalbumin and anti-ovalbumin, in complete Freund's adjuvant (123). Other methods that have been used include immunization with antibody agglutinated suspensions of Proteus vulgaris (38) and subcutaneous injection

in adjuvant of isolated IgG, or IgG cross-linked with glutaraldehyde to enhance immunogenicity (25). Antisera must then be tested for specificity to ensure it reacts with the immunizing allotype and not with any other immunoglobulin allotype. Alternatively, antiserum to rabbit immunoglobulin allotypes may be raised in another species. This antiserum must, however, be absorbed prior to use with rabbit immunoglobulin which lacks the allotype in question in order to remove anti-isotype activity.

Detection methods employed in early investigations of rabbit allotypy primarily involved precipitin tests in agar gels (31 - 33, 41, 44, 123 - 126). The Oudin and Ouchterlony techniques have been widely used for quantitative and qualitative studies. The former method is generally used for quantitative studies provided a pure standard is available, while the latter method is normally reserved for qualitative studies. Although radioimmunoassay techniques have mostly replaced quantitative gel diffusion methods, the Ouchterlony double diffusion assay remains popular for qualitative investigations.

The radioimmunoassay technique was introduced in 1963 by Dray and Nisonoff (30). This method greatly enhanced the sensitivity of quantitative as well as qualitative assays, and soon many variations of the technique were developed. One popular method (5) involves ethylchloroformate (ECF) solidification of antiallotypic antisera and the use of these antisera in binding or binding inhibition tests where the antigen has been radiolabeled (167). A similar method was developed by Kindt

(78) in which a shorter preparation time and smaller amounts of antisera are required. This method involved coupling of whole antisera or specifically isolated antibody fractions to Sepharose beads which have been activated with N-hydroxy-succinimide (57). The activated Sepharose (HAS) may then be stored as a lyophilized powder (23, 24) for later use. The dry material is reacted with the whole antiserum or specific antibody in aqueous solution at neutral pH. The reaction is stopped by addition of excess glycine, and the product is washed. Solidified antisera prepared in this manner retain much of their original activity, and no losses of fine specificity have been detected (57).

More recently, a radioimmunoassay technique has been developed by Rodkey and Braun (144) which is particularly well suited for studies of rabbit immunoglobulins. This method involved incubation of aliquots containing 1-10 mg of ^{125}I-labeled F(ab')$_2$ fragments with antiallotype antisera at room temperature for 2-4 hours on a rotating platform. Complexes of antibody and antigen are precipitated by adding polyethylene glycol (PEG) 6000 in borate-saline to a final concentration of 18%. This system appears to selectively precipitate intact IgG, while unbound F(ab')$_2$ fragments remain in solution.

C. Allotypic Groups Associated with Rabbit Immunoglobulin Molecules

Genetic polymorphism or allotypy associated with rabbit immunoglobulins has been observed in L-chains of both the κ

and λ types, in the C_H regions of IgG, IgM and IgA, in the carbohydrate moieties, and in the secretory component of IgA. In addition, a distinct set of allotypic markers is localized in the V_H regions which are common to all rabbit H-chains studied (38, 73, 87, 101, 126, 165). The V_H and C_H genes thus far identified are closely linked, and certain combinations of V_H and C_H markers (designated phenogroups or haplogroups) are preferentially associated in rabbit populations (39, 90, 106). Most studies are based on small laboratory populations or on colonies bred from a small number of rabbits. Such a small sampling of the rabbit population may well be the reason for the limited number of phenogroups thus far observed, and this could be significant in that the existence of phenogroups is one piece of evidence for the close linkage of all genes controlling the various classes of H-chains.

The rabbit immunoglobulin allotypic groups reported thus far include groups a, x and y located in the V_H regions, groups d and e, and A8 and A10 located in the C_H regions of gamma H-chains, groups f and g located in C_H regions of alpha H-chains, groups n and Ms located in the C_H regions of mu H-chains, group t located in the secretory component of IgA, group sg found on the carbohydrate moiety, and groups b and c located on the kappa and lambda L-chains respectively (90).

1. V_H allotypes of group a. The occurrence of allotypic determinants in the V_H region of immunoglobulin molecules appears

to be a unique characteristic found only in lagomorphs. These a locus determinants are particularly interesting in that they provide a built-in probe with which to investigate the regulation and control of antibody synthesis at the genetic level.

There were three alleles, a1, a2 and a3, originally reported by Oudin (124) from studies involving domesticated rabbits which had probably been selected from a small sample of wild rabbits belonging to the subspecies Oryctolagus cuniculus cuniculus (10). Recent studies involving wild rabbits of the subspecies O. cuniculus cuniculus have revealed two additional a locus alleles, a100 (12, 15) and a101 (12), suggesting a much more complex system than originally thought.

The original group a allotypes (124) were shown to be present in the Fd region of the H-chains by their absence on L-chains (157) and by their presence on the Fab or Fd piece (71, 115). Subsequent work has further localized these specificities to the V_H region by the direct demonstration of these epitopes on peptides (105, 117).

2. V_H allotypes of groups x and y. Several studies have shown that a small percentage of rabbit immunoglobulin molecules lack group a allotypic determinants. Work by Dray and Nisonoff (30), and Stemke (158) revealed that 10-30% of IgG samples from individual rabbits did not react with group a antisera. Subsequent investigations (26, 168) were able to show that these a-negative populations could be enhanced in serum of rabbits subjected to homozygous allotype suppression in zygote transfer

experiments (section F). Additional evidence of V_H regions lacking group a determinants was provided by Kindt et al. (88). These investigators elicited a homogeneous antibody to streptococcal carbohydrate which lacked all known group a determinants.

The question arises as to whether these a-negative populations represent unknown group a allotypes or if there exists one or more additional allotypic groups within the V_H region. Several lines of evidence supporting the latter possibility are discussed by Kindt (78). He indicated that in all breeding studies reported, group a allotypes behave as codominant autosomal alleles and that deviations would be observed if alleles existed for which no typing sera were available. In addition, a-negative molecules are present in sera from rabbits heterozygous for the a allotypes (170). Findings by Tack et al. (162) that a-negative molecules can be distinguished by their amino acid composition and by the sequence of amino terminal peptides weakens the suggestion that allotypic specificities are simply masked on a-negative molecules.

Finally, serological evidence is available that indicates a-negative molecules represent at least two allotypic groups separate from group a. Antisera have been developed by Knight et al. (89) and Kim and Dray (73, 74) against allotypes 32 (group x) and 33 (group y). These allotypes have been shown to exist on distinct sets of IgG molecules isolated from allotypically suppressed rabbits (74).

3. <u>CY allotypes of groups d, e, A8 and A10.</u> Mandy and Todd (109) used hemagglutination assays to demonstrate allotypic specificity

in the C_H region of rabbit IgG molecules. This specificity has been localized to the hinge region of IgG by showing its presence on $F(ab')_2$ fragments but not on Fab (110), and is designated d11. Shortly following, an allele, d12, was found (111). Heavy chains with all combinations of a and d allotypes have been demonstrated although some combinations such as a2d11 are rare (82, 111).

Other studies by Dubiski (35, 36) reported a second allotype, designated e14, in the Cγ region in addition to an allele e15. Since the C_H segment of the H-chain is assumed to be controlled by a single gene the d and e determinants are considered to be products of a single locus designated de.

Two other allotypes have been observed in the Cγ region but have not yet been assigned to loci (58). These specificities are designated A8 and A10 and have only been observed on H-chains bearing the a1 allotype. There is some speculation that variation in carbohydrate prosthetic groups may be responsible for these epitopes rather than variation in primary structure.

4. Cα allotypes of groups f, g and t. Two groups of allotypes have been found in association with rabbit IgA molecules (21, 22, 95). Epitopes controlled by the f locus include f69, f70, f71, f72 and f73, while the g locus controls epitopes g74, g75, g76 and g77. Two additional allotypic specificities, t61 and t62, are expressed on the secretory components of sIgA and appear to be controlled by allelic genes at the t locus (92).

The f and g allotypes are closely linked to one another and to allotypes of group a, while the t allotypes of the secretory component are not linked to allotypes of the H- or L-chains (92). Also, of the 20 potential combinations between the five group f and the four group g allotypes only five have been observed (78).

5. Cμ allotypes of groups n and Ms. The alleles N^{81} and N^{82}, at the N locus control allotypic specificities N81 and N82, which presumably occur in the C_H region of IgM molecules (54). Several other IgM allotypes have been observed by Kelus and his collaborators and are designated Ms1, Ms2, Ms3, Ms4, Ms5 and Ms6 (70, 72, 149). These specificities have not yet been assigned to a genetic locus.

6. L-chain allotypes of groups b and c. Among the six allotypes first described by Oudin (124) were three group b allotypes designated b4, b5 and b6. These were shown to be on the κ L-chains by Stemke in 1964 (157). Soon, an additional specificity designated b9 was added to this group (13, 42). More recently, a fifth allele, $b4^{var}$ has been detected by sequence and genetic studies (154, 155). As compared to b4, $b4^{var}$ is characterized by the presence of serine in place of alanine at position 121 and leucine in place of glutamine at position 124. These specificities of the group b allotypes have all been documented through studies involving domesticated rabbits. Recent investigations of wild rabbits of the subspecies Oryctolagus cuniculus algirus (10) have revealed four new allotypic

specificities of the b series, A93, A94, A95 and A96. The A95 specificity has been most extensively studied and genetic data indicate that it is under control of a b locus allele and can thus be designated b95 (6).

A small percentage of rabbit immunoglobulin molecules carry L-chains of the λ type which do not express the group b allotypes. Comparative studies of this L-chain type (3, 18, 139, 140) have been made possible through homozygous suppression experiments (section F) used to increase the serum concentration of λ L-chains. Two markers designated c7 and c21 have been described for this L-chain type (108). These were initially thought to be alleles until subsequent breeding studies yielded conflicting results (55). When two animals each of the phenotype c7c21 were mated the expected proportions of homozygotes (25% each) and heterozygotes (50%) were not observed. Instead, all the animals were c7c21 suggesting that c7 and c21 are closely linked and not encoded at homologous DNA regions. Since other breeding experiments (108) have yielded expected results it is possible that both allotypes c7 and c21 have true alleles that are not being detected by the antisera (78).

D. Structural Correlates

The allotypes for which structural data is available include those of groups a, b, d and e. Allotypes of groups d and e involve small, well characterized differences in amino acid substitution, while those of groups a and b involve multiple, less well defined

amino acid interchanges thus suggesting greater genetic complexity.

1. Group a correlates. Amino acid composition studies have shown differences among IgG H-chains of different allotypic specificity but of the same antibody specificity (93). Compositional analyses were done on purified anit-p-aminophenylarsonic acid antibodies isolated from either a1b4 or a3b4 rabbits. The amino acids for which significant compositional differences were found and numerical values reported as a3 minus a1 are as follows: Arg (-20), Asp (-2.0), Thr (-6.0), Pro (-3.0), Ala (+4.9), Val (-3.0), Ile (-2.3) and Phe (+2.4). The system of three-letter symbols used to designate the amino acids is described by Lehninger (100). Furthermore, the same differences could be shown among IgM H-chain molecules from the same animals. Similar results were obtained in studies on Fd fragments from IgG pools (64).

Subsequently, differences were demonstrated among isolated amino-terminal peptides from H-chains of total IgG fractions from rabbits with different group a allotypes (172). Allotype-related sequence differences were shown to be present in the N-terminal 34 residues of a1 and a3 IgG H-chain polypeptides from homozygous animals. Differences were observed at 12 positions including 3 Ser, 4 Val or Leu, 10 Arg, 13 Thr, 15 Thr, 16 Pro, 17 Gly, 24 Val or Ala, 28 Ser, 29 Leu, 32 Tyr and 33 Ala or Asp for a1 molecules, and 3 Ser or Gln, 4 Leu, 10 Val, 13 Lys, 15 Gly, 16 Ala, 17 Ser, 24 Ala, 28 Asn, 29 Gly or Ala, 32 Phe or Tyr and 33 Tyr for a3 molecules. It was also shown that IgA H-chain

peptides were similar to IgG peptides of the same allotype (173).

Two major areas of possible allotypic differences were revealed from sequence studies conducted on H-chains from pooled IgG of allotypes a1 and a3 (118), and a partial V_H sequence of an antibody with allotype a2 (49). These data together with subsequent sequencing data (65, 66, 134) suggest that valid correlates of group a allotypes occur at positions 4, 7, 9, 11, 12, 15 and 16 near the amino terminus and at positions 84 and 85. The amino acid substitutions at these positions are 4 Cys, 7 Gly, 9 Arg, 11 Val, 12 Thr, 15 Pro, 16 Gly, 84 Thr and 85 Glu for a1 molecules, 4 Lys, 7 Glu, 9 Gly, 11 Phe, 12 Lys, 15 Asn, 16 Thr, 84 Ala and 85 Gln for a2 molecules and 4 Glu, 7 Gly, 9 Asp or Val, 11 Val, 12 Lys, 15 Ala, 16 Ser, 84 Ala and 85 Ala for a3 molecules.

In addition, there may be group a allotypic correlates at positions 69 - 73. The a1 and a3 H-chains have identical sequences in this region whereas a homogeneous a2 antibody has a considerably different sequence (50). The sequence for a1 and a3 molecules is Phe - Thr - Ile - Ser - Lys at positions 69 - 73, whereas the sequence encompassing these residues for a2 molecules is Ser - Thr - Ile - Thr - Arg.

Although the well-documented correlates of the group a allotypes (amino-terminal substitutions and positions 80 - 85) are distantly separated along the peptide chain, three dimensional models of IgG derived from X-ray chrystallographic studies (133) demonstrate close proximity of positions 15 - 17 and 84 - 85.

It has been shown that disruption of the intra-chain disulfide bridges of H-chains may cause a loss of group a allotypic specificity (105) thus suggesting that both differences must be present in close proximity for expression of the allotypic determinant.

Although no structural data are available that identify specific structural correlates of groups x and y, there are data on general structural aspects of the a-negative H-chains. This evidence serves mainly to negate any possibility that a-negative molecules represent unidentified subclasses of IgG. Studies involving peptide mapping of Fcγ fragments revealed no differences for a-positive and a-negative chains (89, 138, 162). Similar results were obtained in comparative studies including CNBr fragments and compositional analysis of a C-terminal octadecapeptide (162).

The only structural differences thus far observed between a-positive and a-negative molecules occur in the amino terminal region of H-chains. Prahl et al. (137) demonstrated differences in amino acid composition between c1 fragments (an H-chain fragment obtained by CNBr digestion equivalent to the Fd region) of a-positive and a-negative chains. These differences do not correlate with those found between H-chains of different group a allotypes (93).

A final characteristic of a-negative molecules that appears to differentiate them from a-positive molecules involves the

amino terminal sequence. The a-positive chains begin with pGlu-Ser-Leu (49, 65, 118, 134), whereas a-negative chains begin with pGlu-Glu-Gln (162, 170, 172, 173).

2. Group d and e correlates. Work by Prahl et al. (136) revealed that group d allotypes correlated with a Met (d11) Thr (D12) interchange at the amino terminal side of the cysteine involved in the inter-H-chain disulfide bond (position 225). Subsequently, McBurnette and Mandy (113) demonstrated that a hinge region peptide including the Met substitution has d11 antigenic activity.

A single amino acid interchange was also implicated for the group e allotypes at position 309 in the Cγ region (2). These allotypes were correlated with Thr (e14) and Ala (e15).

3. Group b correlates. Attempts to localize group b structural correlates has been difficult primarily due to the problem of separating L-chain variable and constant domains without reducing the interdomain disulfide bond which links these regions (159). Although many biochemical and genetic studies suggest that these domains are encoded by separate regions of DNA it remains unclear whether the group b allotypes occur in the variable half or the constant half of the κ L-chains (45).

Several studies have documented amino acid substitutions in the V_H region which appear to correlate with allotypic variation. Waterfield et al. (169) have described quantitative differences in the expression of amino terminal V_H sequences in

L-chains of different allotypes. Thunberg et al. (164) and Fraser et al. (52) have shown the presence of numerous b9 associated substitutions also in the V_H region.

Peptide fragment studies involving residues 210 - 214 located in the C-terminus of κ L-chains have shown differences among the b4, b5 and b6 allotypes (4, 51). The sequences at these positions are Asn - Arg - Gly - Asp - Cys for b4 chains, Ser - Arg - Lys - Asx - Cys - for b5 chains and Ser - Arg - Lys - Ser - Cys for b6 chains. Subsequently, Goodfleisch (56) characterized a similar peptide from b9 and found it to be identical to b4. Sequencing data of peptides containing residues 134 Cys and 194 Cys which make up the intra-C-region disulfide bridge show perhaps a single residue difference among the b9, b4 and b5 allotypes with no differences occurring around position 134 (17, 56, 94). More extensive variation was found by Poulson et al. (135) around residue 171 Cys which participates in the interdomain bridge. The sequence for b4 molecules involving positions 163 - 171 is Lys - Thr - Pro - Glu - Asn - Ser - Ala - Asp - Cys. The single amino acid substitution observed in b5 molecules is 169 Asp, and substitutions for b9 molecules include 163 Thr, 167 Ser, 168 Pro and 169 Glu.

Recent studies have revealed a large degree of amino acid substitution in the C-region of κ L-chains (46, 160, 175). Results from these investigations involving b4, b6 and b9 chains show that differences up to 35% exist between these various

allotypes. Also, a small number of substitutions appear to distinguish constant regions of the same allotype involving b4 (154) and b6 (45).

E. Allotype Subspecificities

Indications of allotype heterogeneity were first found when many investigators observed double lines in gel diffusion patterns between rabbit sera and allotypic antisera (33, 126 - 128). This phenomenon suggested that multiple forms of allotypes existed.

Kakinuma (69) identified two subpopulations of a2 molecules that comprise 40-60% of the total a2 molecules in a homozygous rabbit. In studies designed to compare group a allotypic determinants expressed on different classes of immunoglobulin molecules Todd and Inman (166) found that the expression of the a2 epitopes of IgM was deficient with respect to that of IgG. Segre et al. (148) conducted similar experiments and found no epitopes of a1 specificity which were present only on IgG and not IgM. However, they found that antibody produced by single cells generally has a greater affinity for the immunizing IgG than for the cross-reacting IgM. A re-examination of the problem by Seto (151 - 153) using gel precipitation methods demonstrated that some epitopes of a1 and a3 allotypic specificities are carried by IgM and not IgG, and that some are carried by IgG and not IgM.

Other observations suggesting that more than one distinct population of molecules is controlled by each a locus allele include cross-reactions between different group a epitopes. Renneboog-Squilbin (141) has described a cross-reaction between some a1 and a2 molecules using anti-a1 antiserum raised in an a3 rabbit. Brezin and Cazenave (11) have shown cross-reactivity between a1 IgG and an anti-a3 antiserum made in a homozygous a2 rabbit. Some cross-reactivity was demonstrated by Roalnd et al. (146) between a small population of a1 molecules and anti-a2 antibodies, made in a homozygous a3 rabbit.

More direct evidence is available for existence of sub-specificities of the a locus determinants. Kindt et al. (85) have reported that antibodies of restricted heterogeneity (122) from a2 and a3 rabbits were incapable of inhibiting completely a reaction of pooled a2 and a3 molecules with anti-a2 or anti-a3 antisera. Horng et al. (62) have purified anti-a1 antibodies of four specificities by absorbing specific antisera, with antibodies of restricted heterogeneity. Using these antibodies they were able to identify three distinct groups of molecules, each with a unique marker and a common marker shared by the three populations. Rodkey and Braun (144) have employed the methods of polyacrylamide gel electrophoresis, isoelectric focusing, agarose block electrophoresis and preparative isoelectric focusing to isolate homogeneous populations of antibodies of the a2 and a3 specificities. Pruified antibodies of the a3 allotype were used as inhibitors in radioimmunoassays involving three different anti-a3

antisera. The data gave evidence for the existence of at least 12 serologically discernible subspecificities of the a3 rabbit allotype. Furthermore, anti-allotype antisera were made against single band preparations found to be deficient for a2 and a3 markers. These antisera were assayed for reaction with pooled a2 or a3 molecules and the results demonstrated, in contrast to the a1 allotype (62), no detectable determinants common to either a2 or a3 molecules.

Rabbit antibody light chains exhibit extensive diversity in the sequence of their amino terminal residues (9, 61, 86) and it has been shown that amino-terminal sequence analysis of κ chains from IgG pools can distinguish between the group b allotypes (61, 164, 169). Kindt et al. (84) have compared a group of homogeneous antibodies by quantitative inhibition of radioprecipitation to determine if these sequence differences have any influence on serological determinants of the group b allotypes. They found no differences in the ability of each homogeneous antibody tested to inhibit a reaction between anti-b4 and a b4 IgG-^{125}I pool even though the inhibitors differed considerably in their amino-terminal sequences. Subsequently, Thunberg and Kindt (163) were able to show at least two b9 allotype subspecificities in a study of five homogeneous antibodies. This finding indicates that the uniform specificity of the b4 allotype may not be applicable for the other group b determinants. In support of this interpretation is the previous finding by Oudin (128) of cross-reactions between allotypes b5

and b6 suggesting the existence of subspecificities associated with these group b allotypes as well.

F. Allotype Suppression

Allotype suppression involves lowering or complete suppression of in vivo synthesis of a particular allotypic specificity by administration of anti-allotype antibodies. Rabbit allotypes appear to be controlled by allelic structural genes on autosomal chromosomes (section H), and in the heterozygous rabbit treated for allotype suppression there is a compensatory increase in synthesis of immunoglobulins bearing the other allotype. In the homozygous rabbit no alternative allele exists and a different L-chain type or an H-chain of a different allotypic group will be synthesized.

Suppression of a heterozygous rabbit was first reported by Dray (28) in a study involving neonatal administration of antibody directed against the paternal allotype. The suppressed allotype did not appear until late in the animals life.

Homozygous and heterozygous suppression are experimentally differentiated by the fact that immunoglobulin bearing the allotype to be suppressed cannot be present in the circulation at the time of anti-allotype injection (104). This complicates the matter of homozygous suppression since maternal allotype is present in the neonate circulation. The problem was circumvented by David and Todd (26), and Vice et al. (168) using the technique

of zygote transfer. Homozygous ova at about the eight-cell stage are transferred to a pseudopregnant female of a different allotype. The offspring may then be successfully suppressed at birth for the allotype of their true parents since this allotype is not present in the circulation. More effective suppression is realized if the surrogate mother is immunized against the allotype to be suppressed.

G. Allelic Exclusion and Selection

Individual antibody producing cells from allotypically heterozygous lagomorphs synthesize immunoglobulin molecules which display only one allotype from a particular group. Early work first suggesting that allelic exclusion exists in rabbit lymphoid cells involved the finding that individual immunoglobulin molecules exhibit only one allotype from an allelic pair for either the bisymmetrical four-chain structure of IgG (30) or for the oligomeric IgA and IgM molecules (99, 147). This apparent selectivity of gene expression has been demonstrated for human myeloma proteins (59), for certain homogeneous antibodies in humans (112) and in rabbits (53, 88, 121, 145).

Studies of rabbit immunoglobulin-producing lymphoid cells reveal that most of these cells display only one allotype from each group (16, 129). Davie et al. (27), using fluorescent antibody techniques and radioiodine markers, have shown that only a small percentage of cells carry two allotypes from the b group.

Similar results were obtained by Pernis et al. (130) using mixed fluorescent labels. In contrast, Sell et al. (150) have demonstrated by blast transformation and thymidine uptake experiments that a significant number of peripheral blood lymphocytes require reaction with two allotypic antisera for stimulation. Mixed anti-globulin reaction studies by Wolf et al. (174) suggest that 50% of circulating lymphocytes from heterozygous rabbits bear two group b allotypes.

Jones et al. (67, 68) studied this problem using the fluorescence-activated-cell sorter (FACS). Lymphocytes from peripheral blood and Peyer's patches of $\underline{b}^5\underline{b}^9$ and $\underline{b}^4\underline{b}^5$ rabbits were stained with rhodamine- and fluorescein-labeled anti-allotype reagents. Preliminary examination revealed that up to 63% of peripheral blood lymphocytes and 15% of Peyer's patch lymphocytes were double stained for the two group b epitopes. Double staining cells were purified by a double pass separation procedure on the FACS, and were treated with Pronase to remove membrane bound immunoglobulin. Membrane immunoglobulin could then be regenerated after a period of incubation in a modified Mishell and Dutton culture medium (60, 116) although the proportion of cells bearing both allotypes was greatly reduced. This suggested that the lymphocytes were restricted to the synthesis of membrane immunoglobulin molecules of a single allotype at a given time.

Other studies in the mouse (171) and the rabbit (63) have demonstrated allelic exclusion with respect to antibody synthesis by single cells. Ferrarini et al. (48) have also concluded from

investigations of rosette-forming cells that only one allelic allotype could be detected per cell.

In later investigations cells bearing two allotypes on their surface, both in the a and b systems, have been demonstrated on rabbit lymphoid cells from the blood, bone marrow and Peyers' patches, and in the cytoplasm of these cells (7, 67, 75, 76, 102, 174). Most recently, Abehsira et al. (1), using methods of plaque-forming cell enhancement and micromanipulation, have generated results suggesting that many lymphoid cells from rabbits heterozygous for the group a determinants secrete immunoglobulin molecules of two types.

The question of whether or not allelic exclusion is the rule for all immunoglobulin allotypes remains unresolved. If allelic exclusion is the rule then immunoglobulins represent the only known system of autosomal gene products where such a phenomenon exists (78). Allelic exclusion has been demonstrated for genes located on the X chromosome (103).

A phenomenon related to allelic exclusion is allelic selection. Work by Spring et al. (156) indicated that allotypic epitopes of immunoglobulins do not influence the specificities of their binding sites. Their results indicate that the portion of the surface of the molecule comprising the antibody-combining site is independent of the region of the surface which combines with anti-allotypic antibody. However, investigations to determine the relationship between allotypes and binding specificity have

shown that certain antigenic stimuli preferentially elicit antibodies bearing a particular allotype.

Although early studies (53, 98, 142) revealed no clear tendency for synthesis of antibodies bearing particular allotypes in response to antigen, a later investigation by Catty et al. (14) demonstrated preferential synthesis of antibody with allotype b4 in b4b5 heterozygous rabbits immunized with pneumococcal polysaccharide. In a few other rabbits the converse was noted. Subsequently, Zimmerman and Haurowitz (176) were able to show nearly complete absence of the a3 allotype in a1a3 rabbits immunized with p-azophenylarsonate. No such allotypic preference could be demonstrated to other haptens in the same rabbits. Kindt (77) has shown there is a preferential response by molecules of the b4 allotype on antistreptococcal antibodies elicited in b4b9 heterozygous rabbits.

Dubiski (37) has examined allelic selection in rabbits heterozygous for the group b allotypes and focuses attention on the unequal proportions of allelic allotypes produced in response to antigen. There is a predictable preponderance of one allotypic specificity over the other in circulating immunoglobulins which Dubiski calls the "pecking order". The order of predominance noted is b4 > b6 > b5 > b9. Furthermore, this preponderance is reflected in the relative number of B cells carrying a particular allotypically marked receptor (19, 20). This hierarchy of expression is also apparent for the L-chain types, where $\kappa > \lambda$ (3), and for the group a and a-negative H-chains

(161). Although no evidence is sited, Kindt (78) claims there is an order of preponderance for the group a allotypes (a1 > a3 > a2) which is less predictable than that for the group b allotypes.

H. Genetic Control of Allotypes

Breeding studies (92, 104) involving rabbit immunoglobulin allotypy have enabled placement of the allotypic groups at one of four unlinked or distantly linked genetic regions: (1) all H-chain allotypes, (2) the κ L-chain allotypes, (3) the λ L-chain allotypes and (4) the secretory piece allotypes. The latter three regions are relatively simple, each controlling a single allotypic group, whereas the H-chain region is quite complex with a large number of allotypic groups.

The H-chain linkage group controls several closely linked genetic loci for allotypic groups in the V_H region and the C_H region of IgG, IgA and IgM. The allotypic groups encoded by these loci are a, x, y, n, d, e, f, and g. A large number of distinct haplotypes is theoretically possible from the many possible combinations of these allotypes, however only a few have ever been observed and are designated haplogroups (106). These haplogroups are analogous to the different haplotypes described for human immunoglogulin allotypes (119).

Dubiski and Good (39, 40) have investigated combinations involving the group a and group e allotypes in randomly bred

rabbit populations. Only twelve of the possible twenty-one genotypic combinations were observed. Complete absence of the gene pair $\underline{a}^3\underline{e}^{14}$ in the population accounted for six of the unobserved genotypes, while a low frequency of $\underline{a}^2\underline{e}^{14}$ was implicated in the remainder. Mandy and Todd (111) point out that the allotypic combination $\underline{a}^2\underline{d}^{11}$ is likewise seldom observed, with one such case reported by Kindt et al. (82) in a rabbit of the genotype $\underline{a}^2\underline{a}^2\underline{d}^{11}\underline{d}^{12}$. Serum from this rabbit was found to contain nearly equal amounts of a2d11 and a2d12 molecules.

Todd has observed that group a allotypes are expressed on both the γ and μ classes of H-chains (165). Feinstein (47) has also reported the common occurrence of group a makers in V_H regions of IgG and IgA. Verification of group a allotype expression on IgM (166) and IgA (80, 101, 132) was later realized using more sophisticated techniques. Finally, with the finding by Kindt and Todd (87) that group a allotypes are also found in association with homocytotropic antibodies it could be concluded that group a determinants are present in the variable region of H-chains of each major class. This observation became known as the Todd phenomenon and was important evidence supporting the two gene - one polypeptide concept postulated by Dreyer and Bennett (34).

Other studies involving the H-chain linkage group have revealed the occurrence of recombinatorial events between V_H and C_H allotypes. The d group (110, 111) and e group (54)

allotypes of C_γ chains are known to be linked to the group a allotypes (36). Thus, the allotypic combinations on the majority of H-chains reflect the parental linkage groups of V_H and C_H allotypes. Kindt et al. (81) have shown that offspring from a mating of which one parent is homozygous a2d12 and the other is homozygous a3d11 present a majority of H-chains with allotypes a2d12 or a3d11. Landucci-Tosi et al. (96) have reported similar results for the group a and group e combination. However, some molecules of the recombinant types are always observed. Recombinations have been reported between group a and group e allotypes (107) and between group a and group d allotypes (79). Mage et al. (106) estimate a recombinatorial frequency of 0.3% between V_H and C_H allotypes. Pernis et al. (131) have used cell staining techniques to arrive at an estimate of 1% for recombinations between V_H and C_H allotypes which closely agrees with the figure from a similar study by Landucci-Tosi and Tosi (97). These findings indicate that IgG H-chains are normally synthesized in cis as opposed to trans fashion.

Recombinations have also been observed between the group a and group g allotypes on IgA molecules (91). The frequency of recombinant molecules observed was 2-3%.

The early study of rabbit allotype inheritance by Oudin (128) suggested that allotypic determinants were encoded by codominant, autosomal structural genes which segregated in simple Mendelian fashion. This concept of allotype genetics

has been challenged by findings implying that allotypes are under the control of regulatory genes. Strosberg et al. (159) have observed the occurrence of three group a and three group b allotypes in a single rabbit. Similar findings have been reported regarding the Gm allotypes of man (143) and the C_H allotypes of the mouse (8). Unexpected allotypes, or latent allotypes have also been shown by Kindt et al. (83) for group a determinants of rabbit immunoglobulins. They report that of 120 rabbits tested 50% of the pre-immune sera displayed latent allotype. Additionally, these investigators were able to show that latent allotype expression is sporadic and transitory over an extended period of time. This observation further supports the notion that allotype expression is under some form of regulatory control.

An early investigation by McClintock (114) involving maize genetics provides a plausable model system for regulatory control of lagomorph immunoglobulin allotypes. This study clearly indicated that a controlling element or gene exists which can modulate genetic expression. Regulation at a number of autosomal genetic loci is observed in the presence of the controlling gene. The modification of phenotypic expression of a particular structural gene apparently results from a transpositional event involving the controlling gene. Therefore, it appears that a single controlling gene may be inserted at any one of a number of different genetic loci to regulate or switch on the particular gene involved.

SECTION III

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LITERATURE CITED

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SECTION IV

MANUSCRIPT: QUANTITATIVE DISTRIBUTION OF THE GROUP a ALLOTYPES IN NORMAL HETEROZYGOUS SERA

Normal serum samples from rabbits heterozygous for the group a allotypes were investigated using a sensitive radioimmunoassay inhibition technique to determine absolute serum concentrations of immunoglobulin molecules bearing a1, a2 or a3 epitopes. Three groups of sera representing each of the possible group a heterozygous combinations were selected for study. Results indicated that a predictable, quantitative difference in allotype expression occurs which is manifested by an ordered preponderance of a1 > a3 > a2. These data provided evidence to support theories that a regulatory control mechanism mediates allotype expression and that allotypes are not encoded by simple codominant, allelic structural genes.

INTRODUCTION

Studies by Oudin (1956 a, b) showed that rabbit immunoglobulin molecules exist in polymorphic forms which can be identified with alloantisera. Oudin used the term "allotypy" to describe this phenomenon and referred to the individual antigenic structures recognized by various alloantisera as allotypes. The original two groups of allotypes (Oudin, 1960) have been expanded to include at least 13 groups representing more than 35 allotypic specificities (Kindt, 1975; Knight 1975). These specificities are distributed among the various structural components of rabbit Ig molecules, and particular allotypic epitopes are known to be associated with κ and λ L-chains, C_H regions of IgG, IgM and IgA, carbohydrate moieties and the secretory component of IgA. In addition, a distinct set of allotypic epitopes is located in the V_H regions which are common to all rabbit H-chains.

Many genetic studies have indicated that rabbit Ig allotypes are encoded by autosomal structural genes that behave as codominant alleles and that segregate in a simple Mendelian fashion (Oudin, 1966; Kindt, 1975). This has been challenged by findings suggesting that a regulatory mechanism controls allotype expression. Deviations from Mendelian behavior has been reported in several systems. Strosberg et al. (1974) discovered the occurrence of three group a and three group b allotypes being

expressed in a single rabbit. Similar findings have been observed involving the Gm allotypes of man (Rivat et al., 1973) and the C_H allotypes of the mouse (Bosma and Bosma, 1974). The existence of pluriallelic individuals expressing unexpected or latent allotypes is strong evidence for the existence of a regulatory mechanism governing allotype expression. Other phenomena that add support to this hypothesis include allotype suppression, auto anti-allotype antibody responses, non-random distribution of allotypic epitopes (pecking order) and apparent somatic recombination between V_H and C_H allotypes which may actually be the result of regulatory error (Strosberg, 1977).

The paper addresses the question of allotype distribution involving the group a allotypes. Chou et al. (1974) have examined the expression of the group b allotypic epitopes. The authors find that the hierarchy of predominance (b4 > b6 > b5 > b9) is attributed to an unequal number of cells bearing allotypically marked receptors. This hypothesis is presented to explain the allotype distribution observed in the indirect plaque-forming cell response involving heterozygous animals immunized with sheep red blood cells. The study was extended by showing that preponderance of b4 over b5 is reflected in the ratio of monoallotypic responders of the b4 and b5 allotypes following immunization of b4/b5 rabbits with a limiting dose of antigen. The literature contains numerous references to a similar hierarchy of predominance involving the group a allotypes

(Abehsira et al., 1979; Kindt, 1975; Strosberg, 1977). However, these reports present neither data nor appropriate literature citations to substantiate claims for a predictable order of quantitative expression of group a allotypic epitopes on circulating Ig molecules. The present paper advances such data and discusses the results as evidence for a regulatory control mechanism governing allotype expression.

MATERIALS AND METHODS

General. Several pre-immune rabbit sera were typed for the a, b and c group allotypes using the standard method of immunodiffusion in agar gels. The group d and group e allotypes were not determined. These sera were collected from rabbits obtained from at least three geographically different areas. Anti-allotype antisera were prepared according to the principles of Oudin (1966) by subcutaneous injection in complete Freund's adjuvant of glutaraldehyde polymerized Ig. These antisera had been standardized by comparison with antisera from two reference laboratories.

Isolation of IgG. IgG pools from homozygous a1, a2 or a3 sera were prepared by sodium sulfate precipitation followed by DEAE cellulose chromatography in columns equilibrated with 0.0175 M sodium phosphate buffer, pH 6.9 (Kekwick, 1940; Levy and Sober, 1960). Solutions containing purified IgG were concentrated to a minimum volume of 10 mg/ml and dialyzed exhaustively against a borate saline buffer solution (BSB)[1] for future use. Quantitation of IgG was done spectrophotometrically based on $E^{1\%}_{1cm} = 15$ at a wavelength of 280nm (Russell and Donahue, 1968).

Radioimmunoassays. Aliquots of IgG were pepsin digested and the $F(ab')_2$ fragments were isolated by gel filtration

[1] 1.0gm boric acid, 8.43gm NaCl and 0.1gm NaOH in 1l of H_2O, pH 8.0.

chromatography on Sephadex G-150 columns equilibrated with BSB. $F(ab')_2$ fragments were radioiodinated with ^{125}I using the chloramine T method (Greenwood et al., 1963). Assay tubes were coated with bovine serum albumin to reduce nonspecific binding of protein to walls. Anti-allotype antiserum was added followed by either 10ng of labeled $F(ab')_2$ or IgG inhibitor followed by 10ng of labeled $F(ab')_2$. All reaction mixtures were allowed to incubate at room temperature for 1h. Antigen-antibody complexes were precipitated by adding polyethylene glycol (PEG) 6000 to a final concentration of 18% and incubating at room temperature for 1h (Rodkey and Braun, 1979). Precipitates were centrifuged, washed and counted in a gamma-well scintillation counter. Results were calculated as percent of trichloroacetic acid (TCA) precipitable counts specifically bound in the immune precipitates. Trapping controls were included using normal rabbit, human or goat sera.

RESULTS

Standard inhibition curves were established for each of the three group a allotypes. In each case a standard antigen-binding assay was set up by adjusting the volumes of anti-allotype antiserum used to bind 10ng of labeled $F(ab')_2$ fragments. Volumes of reagents were used that bound 80-90 percent of TCA precipitable counts. Unlabeled IgG, homozygous for the allotype being assayed, was used to prepare a standard inhibition curve for each of the three group a allotypes. Anti-allotype antiserum and inhibitors were mixed and allowed to incubate 1h at room temperature before labeled reference $F(ab')_2$ was added. Trapping controls and TCA controls were included in each assay. Results were calculated as a percent of the uninhibited control. The standard inhibition curves are shown in Figs. 1-3.

Experimental sera were examined for content of group a allotype by using serial dilutions of each serum to inhibit the standard reaction. Dilution steps were made to yield at least two values that could be analyzed on the standard inhibition curve. Concentrations of inhibiting protein contained in the experimental sera were determined by adjusting values extrapolated from the standard curve using appropriate dilution factors. Figures presented in this report represent average concentrations derived from two or more dilutions of each test serum.

Results obtained for heterozygous a1/a2 sera appear in Table 1. Concentrations of Ig molecules bearing the a1 allotype

range from 1.5 to 4.9 mg/ml with only one of the nine sera tested having a concentration above 3.5 mg/ml. Concentrations of the a2 Ig molecules range from 0.5 to 1.7 mg/ml. The ratios of a1 molecules to a2 molecules are relatively consistent with six of the nine occurring between 3.0 and 3.5. Two ratios below this range are 2.7 and 2.1, and one above is 5.1. It is evident from these data that a1 molecules predominate over a2 molecules by a factor of about three to one.

Table 2 shows results for the heterozygous a1/a3 sera. Molecules of the a1 allotype occur in concentrations from 1.9 to 11.0 mg/ml and a3 molecules from 0.9 to 3.2 mg/ml. Ratios of a1 to a3 were found to span a range from 0.9 to 5.2. Only one of the ten sera tested exhibited a ratio below 1.0, while five sera had ratios between 1.0 and 2.0 with the remaining four sera having ratios above 2.0. It may be concluded that a1 generally predominates over a3 in normal a1/a3 sera.

Data representing the final combination of a2/a3 is presented in Table 3. The concentrations and ratios were found to be very consistent. Molecules of the a2 allotype occur in concentrations from 0.4 to 2.1 mg/ml with only one of the eight sera tested having a concentration above 0.8 mg/ml. Similarly, a3 molecules were found to span a concentration range from 1.2 to 5.8 mg/ml with only one serum having more than 2.7 mg/ml of a3 bearing Ig molecules. The ratios of a2 to a3 for seven of the eight sera were 0.3, with a ratio of 0.4 for the remaining

serum. It is clear from the data that a3 Ig molecules predominate by a 3-fold margin over a2 molecules in normal serum samples from a2/a3 heterozygous rabbits.

In summary, the data indicate that a predictable, non-random distribution of the group a allotypes exists in serum from normal, heterozygous rabbits. An order-of-predominance or pecking order of a1 > a3 > a2 is evident with some possible exceptions in a1/a3 sera which may occasionally contain equal amounts of these two allotypes.

DISCUSSION

The method of radioimmunoassay (RIA) inhibition is a sensitive technique which can be controlled to yield accurate results when used to make quantitative measurements. The method as designed for this study employed standard reactions inhibitable with nanogram quantities of protein to determine the concentrations of Ig molecules expressing particular group a epitopes in normal sera from heterozygous rabbits. It should be pointed out that variation in amounts of inhibitor required to establish the three inhibition curves (Figs. 1-3) probably reflects the differences in avidity of the three alloantisera.

The precipitating system used in the RIA inhibitions is particularly well-suited for serological investigations of rabbit immunoglobulins. Intact Ig molecules but not $F(ab')_2$ fragments are selectively precipitated in an 18% PEG 6000 solution. Therefore, in a reaction system of this type any labeled $F(ab')_2$ fragments bound to antibody will be detected in the precipitate, while unbound $F(ab')_2$ remains in solution. This system precludes the necessity of using a secondary antiserum such as goat anti-rabbit Fc to precipitate immune complexes. Consequently, fewer controls and shorter incubation times are required which eliminates certain elements of potential error.

The results demonstrate a predictable, ordered distribution of the group a allotypes in normal serum from heterozygous

rabbits. Both the a1 and a3 molecules, when expressed in combination with the a2 molecules, predominate by a 3:1 margin, whereas in the a1a3 combination, a1 exhibits an average predominance of about 1.5:1 over a3. The order of predominance thus observed for the group a allotypes may be expressed as a1 > a3 > a2. This expression is not common for codominant allelic genes of autosomal chromosomes. Such genes are normally expected to be more randomly expressed leading to an evenly distributed occurrence of their products. The data therefore corroborates a large body of evidence which seriously questions traditional concepts of rabbit Ig allotype genetics.

One concept based on this evidence is that allotypic determinants of a particular group are products of structural genes arranged in tandem on a single chromosome and that allelic components of the system reside in inherited regulatory genes (Yarmush et al., 1979). The most convincing evidence in support of this concept is the existence of unexpected or latent allotypes. Strosberg et al. (1974) first documented this phenomenon in a rabbit hyperimmunized with Micrococcus lysodeikticus. Preimmune serum from this animal contained allotype specificities a1a3, b4b5. Following immunization epitopes a2 and b6 were additionally detected. Mudgett et al. (1975) used a sensitive RIA inhibition method to show that 50% of nonimmune rabbit sera express low levels of group a allotypes not predictable by qualitative typing or breeding data. In a follow-up study to Strosberg's (1974)

original work Mandy and Strosberg (1978) confirmed the presence of latent a2 and b6 molecules in post-immune bleedings and extended the investigation by showing the occurrence of a latent d11 allotype in addition to the nominal d12 and e15 allotypes observed in pre-immune bleedings. Mudgett-Hunter et al. (1978) have reported results from serological characterizations comparing nominal and latent group a epitopes. Their data indicate that sera, IgG preparations and Fab fragments containing either nominal or latent allotypes inhibit standard reactions equally as well suggesting that the full spectrum of allotypic specificities is expressed in latent allotypes. Yarmush and Kindt (1978) have demonstrated the occurrence of latent group b allotypes in pedigreed rabbits. This study reveals the presence of b5 and b9 epitopes in sera from an extended family of homozygous b4 rabbits. Serological comparison of these allotypes with nominal representatives revealed no differences. McCartney-Francis and Mandy (1979) were first to report results from structural comparisons of nominal and latent allotypes. Nominal b4 and b9 L chains contain an aspartic acid-proline peptide bond at residues 109-110 which is cleaved by limited acid hydrolysis to yield two half-light chains. These investigators found that acid hydrolysis of latent b4 L chains gave products identical to those obtained by similar treatment of nominal b4 L chains. In a recent study Yarmush et al. (1980) have presented the first partial sequence analysis of a latent allotype. Latent b9 L chains were isolated

from serum of a b4b4 pedigreed rabbit and were shown to have a partial constant region sequence identical to nominal b9 chains.

Latent Ig allotypes have also been demonstrated in species other than the rabbit. Lobb (1968) has shown the presence of cells bearing Gm allotypic epitopes not predicted by serum phenotype, Rivat et al. (1973) have detected unexpected Gm allotypes in mixed lymphocyte cultures, and Pothier et al. (1974) have detected a Gm factor in a human lymphoid tumor line serially transplanted in neonatal hamsters that was not present in the donor phenotype. Latent allotypes have also been shown to occur in a congenic partner strain of BALB/c mice (Bosma and Bosma, 1974), in SJL/J inbred mice (Weiler and Kolb, 1979) and in the rat (Hunt and Duvall, 1976).

The extensive amount of data in support of latent allotypy and the evidence that these epitopes are structurally identical to nominal epitopes strongly suggests that individual rabbits may possess genetic information for all the epitopes of any given allotype group. In addition, knowledge that there is a predictable, quantitative difference in the occurrence of allotypic determinants in the sera of heterozygous individuals lends support to the idea that a regulatory mechanism is governing the expression of the structural genes.

Deviation from classical Mendelian genetics is found in other polymorphic systems. Anomolous behavior of purported alleles at the histocompatiability locus has prompted Bodmer

(1973) to propose a mechanism for genetic control. As support for his model Bodmer cites the apparent derepression of Tla genes in leukemia cells of the mouse (Boyse et al., 1969). The Tla locus is closely linked to H-2 and controls expression of TL antigens normally found only on thymocytes. All mice except those of thymocyte phenotype Tl. 1, 2, 3 carry repressed Tla genes which are derepressed in leukemic cells. More recently, Garrido et al. (1976) have presented evidence that H-2 specificities of foreign haplotypes appear on the surface of chemically induced mouse tumor cell lines following vaccinia virus infection. A sarcoma induced by 3-methylcholanthrene was passaged in syngeneic BALB/c mice of the $H\text{-}2^d$ haplotype. After seven days peritoneal tumor cells were found to express ten private and three public epitopes normally present only in mice of other H-2 haplotypes. This kind of anomolous behavior in polymorphic systems originally thought to be controlled by simple allelic structural genes suggests that genetic regulatory mechanisms may be operative in more eucaryotic systems than previously thought.

Other reports lend indirect but significant support for existence of an inherited regulatory mechanism controlling rabbit allotype expression. Extensive amino acid sequence differences exist among Ig molecules bearing the group a (Jaton and Braun, 1972; Fleischman, 1971; Mole et al., 1971; Porter, 1974) and group b (Farnsworth et al., 1976) allotypes. Allotype

subspecificities occur in single animals suggesting that several forms of a given "allele" co-exist on the same chromosome. Rodkey and Braun (1979) have suggested the existence of at least twelve serological subspecificities within the a3 allotype. A relatively high frequency of apparent recombinatorial events between V_H and C_H allotypes has been demonstrated (Mage et al., 1973; Pernis et al., 1973; Knight et al., 1974) which suggests a trans versus cis expression of allelic genes. However, this may be a manifestation of aberrant function of a regulatory gene controlling the expression of tandemly arranged structural genes on a single chromosome. The phenomenon of allelic exclusion (Jones et al., 1973; Jones et al., 1974) in which only one gene of an allelic pair is expressed at any given time in a single cell is unprecedented for codominant allelic systems involving autosomal chromosomes (Kindt, 1975). Finally, studies on both latent group a and latent group b allotypes have shown that serum levels of unexpected allotypes are transitory and sporadic (Yarmush et al., 1979).

The data presented thus far makes untenable any suggestion that rabbit Ig allotypes are simply products of codominant allelic genes. There clearly must be a more complex genetic mechanism governing rabbit allotype expression which includes some sort of regulatory mechanism. A central question in this regard is in what way(s) might regulation be effected. It is quite possible that regulatory control, if present, would involve a multi-

component network operating at different levels of protein synthesis and expression. One example includes regulation at the intercellular level. This possibility is made tenable by works demonstrating allotype suppression and the probable occurrence of auto antiallotype antibody production. Dray (1962) first demonstrated that alloantisera could induce suppression of paternally contributed allotypes in heterozygous rabbits. In recent studies Lowe et al. (1975), Horng et al. (1977) and Dubiski and Good (1979) have presented evidence that allotype suppressed rabbits can mount an immune response against the suppressed allotype upon immunization and that this apparent auto anti-allotype response is responsible for the long-term suppression observed in these animals. Consequently, a network theory similar to that of Jerne's (1974) for idiotype regulation is a possibility.

If such a form of intercellular regulation exists then there must be some sort of intracellular controlling mechanism upon which this higher level of regulation operates. The most tractable point of control is at the genetic level or level of transcription. An attractive model may be derived from the regulatory system governing the synthesis of β-galactosidase in E. coli which was presented by Jacob and Monod (1961) as a mechanism for gene regulation in procaryotes. In this model structural genes are controlled by a contiguous segment of DNA called the operator which is in turn controlled by a repressor protein encoded by a

regulator gene at some other locus. The repressor protein interacts with the operator to inhibit transcription. However, blocking of transcription is prevented in the presence of enzyme substrate which modulates the action of the repressor protein. Allotype expression may be similarly controlled at the genetic level by products of a second messenger system which modulate the activities of either repressor proteins or promotor elements. Alternatively, allotype regulation at the genetic level may involve inter- or intrachromosomal recombination of controlling elements. Evidence for transposable genetic control elements in eucaryotes was first reported by McClintock (1956) from extensive studies of maize genetics. Placement of these genetic elements next to a particular gene locus via a transpositional event altered gene expression which was detected by its effect on pigmentation.

In conclusion, there is a large body of evidence to support the suggestion that the predictable, quantitative differences observed in serum levels of rabbit Ig allotypes is the result of a regulatory mechanism controlling phenotypic expression of tandomly arranged structural genes, and that the allelism predicted from the earliest allotype studies may arise from inherited regulatory genes.

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Figure 1. Standard inhibition assay of ^{125}I-labeled a1b4 $F(ab')_2$ with anti-a1 antiserum by increasing amounts of normal pooled a1b4 IgG.

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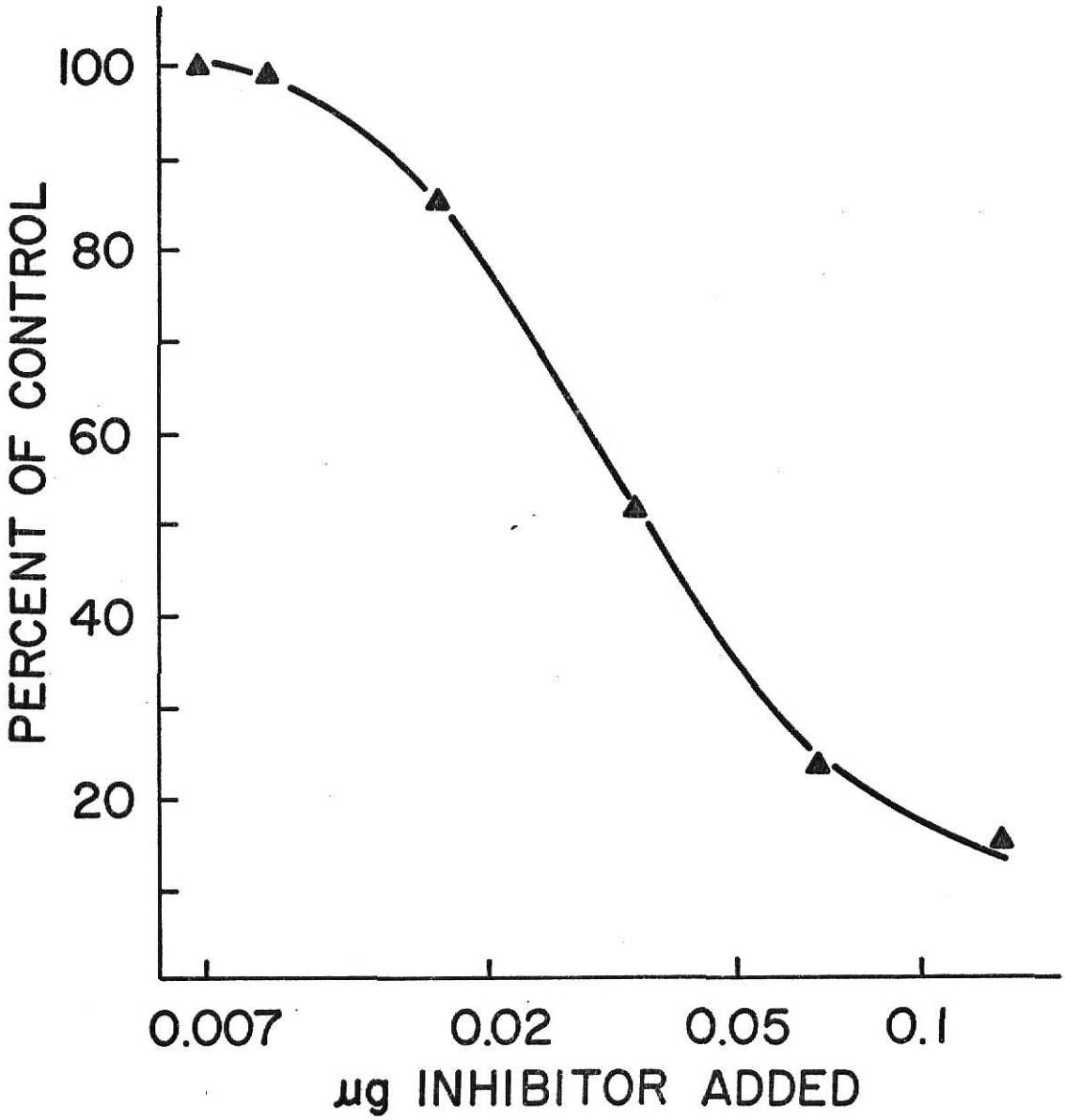
100
80
60
40
20
PERCENT OF CONTROL
0.007
0.02
0.05
0.1
µg INHIBITOR ADDED

Figure 2. Standard inhibition assay of ^{125}I-labeled a2b4 F(ab')$_2$ with anti-a2 antiserum by increasing amounts of normal, pooled a2b4 IgG.

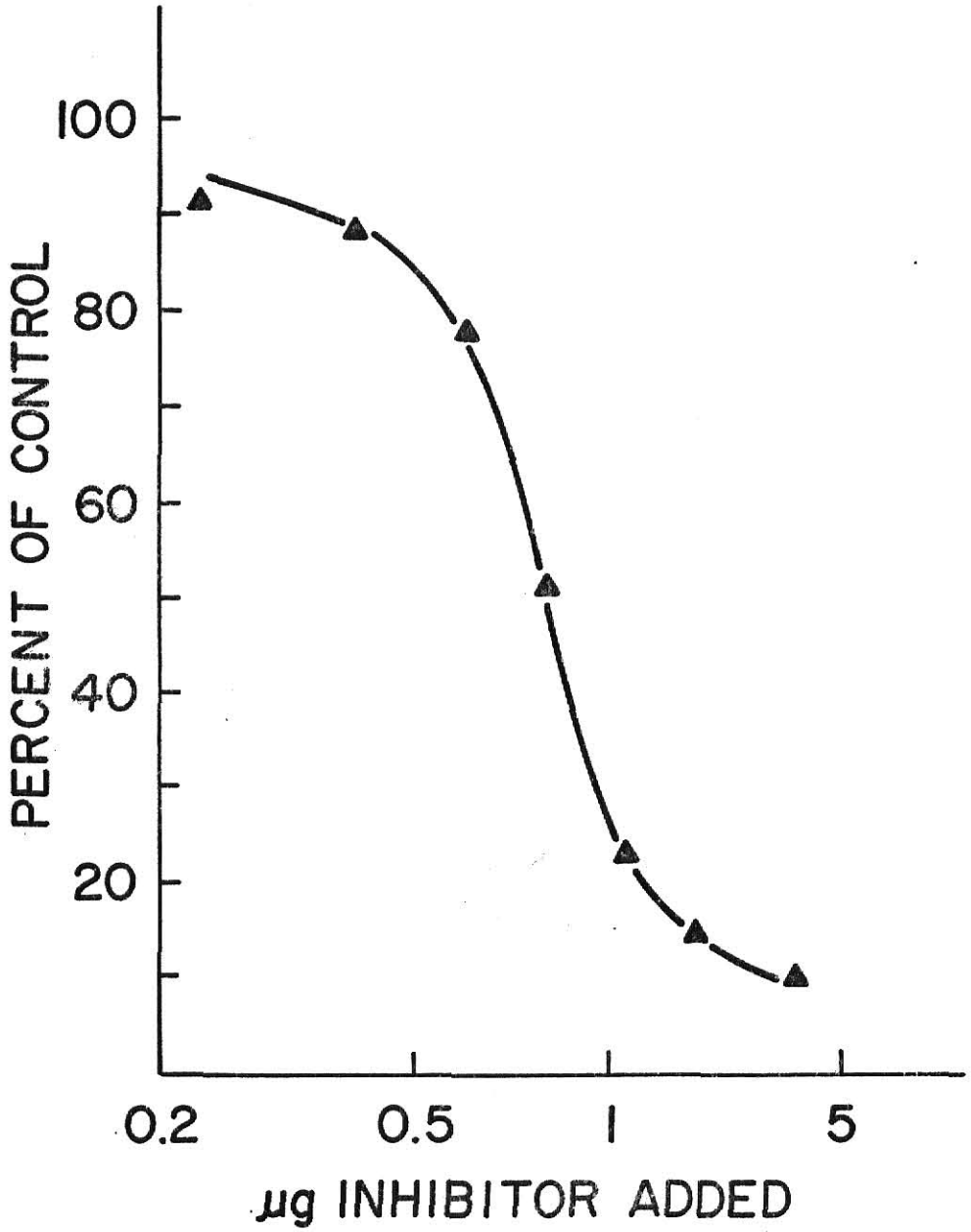
100
80
60
40
20
PERCENT OF CONTROL
0.2
0.5
1
5
µg INHIBITOR ADDED

Figure 3. Standard inhibition assay of ^{125}I-labeled a3b4 F(ab')$_2$ with anti-a3 antiserum by increasing amounts of normal, pooled a3b4 IgG.

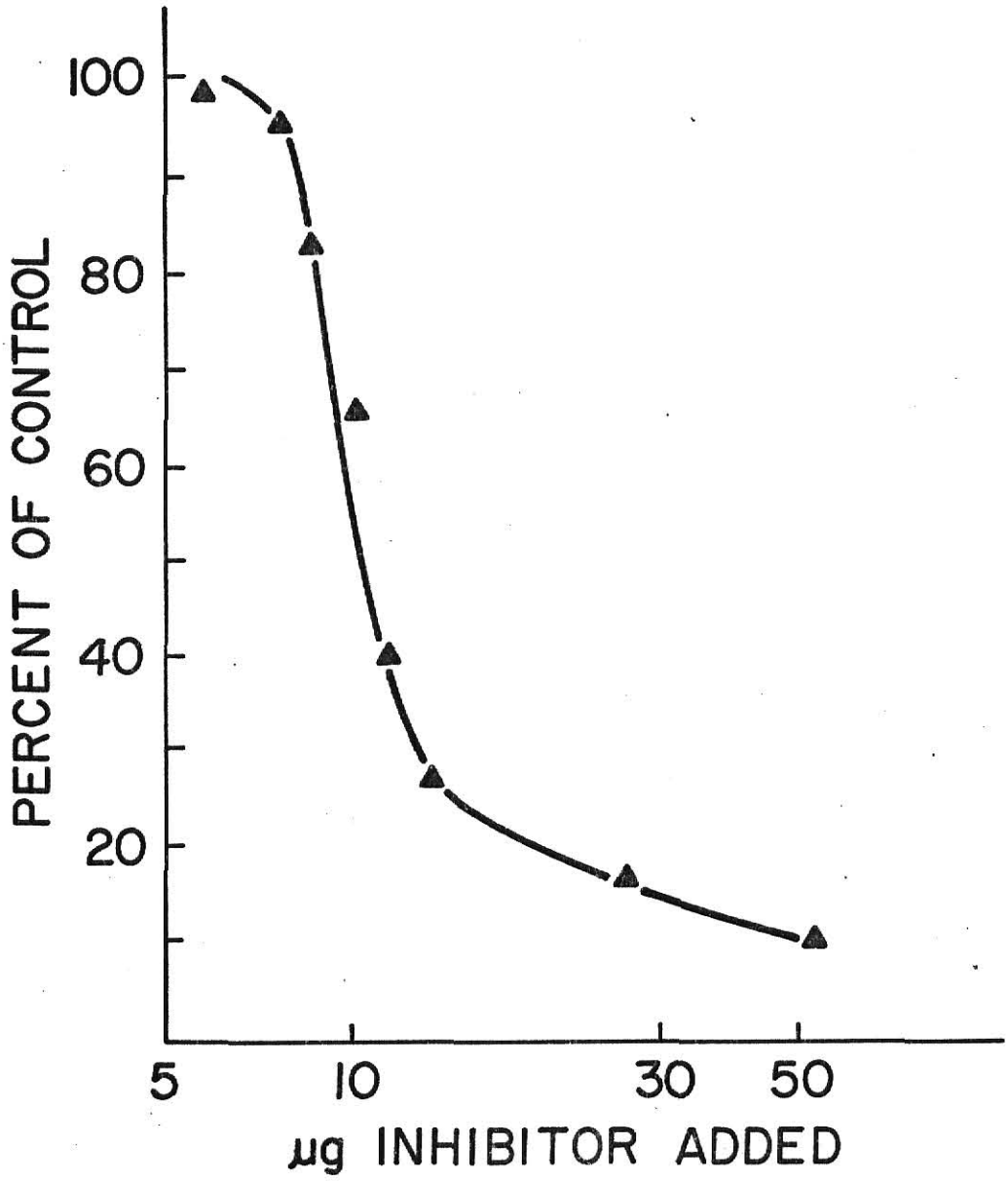
100
80
60
40
20
PERCENT OF CONTROL
5
10
30
50
µg INHIBITOR ADDED

TABLE 1. Allotype Distribution of a1 and a2 Molecules in a1a2 Heterozygous Sera.

Serum No.	Concentrations (mg/ml) a1	a2	Ratios a1/ a2
T-338	2.0	0.6	3.4
T-341	1.5	0.5	3.2
T-344	1.6	0.5	3.5
T-347	1.5	0.5	3.0
47	3.1	1.0	3.0
49	2.8	0.9	3.2
56	2.3	0.8	2.7
191	4.9	1.0	5.1
276	3.5	1.7	2.1

TABLE 2. Allotype Distribution of a1 and a3 Molecules in a1a3 Heterozygous Sera.

Serum No.	Concentrations (mg/ml) a1	a3	Ratios a1/a3
97	4.2	3.2	1.3
98	2.8	2.8	1.0
99	2.8	3.1	0.9
103	3.6	2.7	1.3
7-7	2.5	1.7	1.5
63-7	11.0	3.2	3.4
94-7	7.6	1.5	5.2
95-7	1.9	0.9	2.2
96-7	2.3	1.8	1.3
97-7	2.1	1.0	2.1

TABLE 3. Allotype Distribution of a2 and a3 Molecules in a2a3 Heterozygous Sera.

Serum No.	Concentrations (mg/ml) a2	a3	Ratios a2/a3
100	2.1	5.8	0.4
359	0.8	2.5	0.3
361	0.6	2.0	0.3
362	0.7	2.7	0.3
T-335	0.7	2.1	0.3
T-337	0.7	2.1	0.3
T-339	0.7	2.5	0.3
T-336	0.4	1.2	0.3

QUANTITATIVE DISTRIBUTION OF THE GROUP a ALLOTYPES IN NORMAL HETEROZYGOUS SERA

by

RICHARD STEWART LOFTS JR.
B.S., Michigan State University, 1978

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ABSTRACT

Rabbit allotypes are antigenic determinants or epitopes on immunoglobulin molecules which represent polymorphic variations in primary protein structure and which can be identified serologically using alloantisera. Allotypic epitopes have served as valuable genetic probes in numerous studies which have provided important fundamental information regarding immunoglobulin synthesis. It is hoped that such studies will one day culminate in the application of more effective immunotherapy techniques to certain medical problems such as ineffective Rh prophylaxis, autoimmune diseases, immune complex disorders and graft versus host reactions. Consequently, development of a complete understanding of allotype genetics and expression could be an important step toward better control of some debilitating human diseases.

Early studies defined two groups of allotypes. Each contained epitopes originally thought to be encoded by allelic structural genes on autosomal chromosomes which segregated in simple Mendelian fashion. Today, at least 13 groups are known to exist representing more than 35 individual epitopes distributed over the rabbit immunoglobulin molecules.

Significant instances of anomolous behavior regarding allotype expression have appeared in recent investigations and challenge classical concepts of allotype genetics. Perhaps the most striking anomoly is the common occurrence of latent allotypes. This finding more than any other has prompted theories

suggesting that individuals possess genetic information for the full spectrum of allotypic epitopes and that inherited allelic elements are in the form of regulatory genes. Some other anomolies supporting a reinterpretation of allotype genetics include multiple amino acid differences among "allelic" products, existence of subspecificities associated with certain epitopes, a relatively high frequency of somatic recombination and unequal expression of allelic forms in heterozygotes.

This study reports findings involving unequal expression of the group a allotypes in heterozygous rabbits. A radioimmunoassay inhibition technique was employed to examine three groups of normal rabbit sera representing each of the three possible heterozygous combinations involving epitopes a1, a2 and a3. Dilutions of these sera were used to inhibit standard reactions. Levels of inhibitor were calculated as a percent of uninhibited control and analyzed on standard inhibition curves to determine absolute serum concentrations of immunoglobulin molecules bearing particular allotypic epitopes. Results clearly indicated the occurrence of a predictable, quantitative difference in group a allotype expression in normal heterozygous sera manifested by an ordered preponderance of a1 > a3 > a2. Thus, it appears that some form of selective, quantitative regulation is controlling allotype expression.