

DIVERSE EXPRESSION OF GROUP a ALLOTYPE SPECIFICITIES
ON THE HEAVY CHAINS OF HOMOGENEOUS
RABBIT ANTIBODIES*

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The close association of the group a allotypic specificities of the rabbit with the variable regions of the different classes of heavy (H)¹ chains poses problems in understanding the nature of genetic control mechanisms (1). The amino acid compositions of the H chain variable (V) regions of homologous IgG and IgM antibodies correlate with their group a allotypes (2). Correlations of allotype with V_H sequences also exist for both IgG (3-5) and IgA (6). While the precise amino acid changes responsible for determining the individual group a allotypes remain unknown, they must exist in close proximity to those structural variations which determine the antigen-binding site and the idiotypic marker. These variations could, in a given homogeneous antibody, modulate the antigenic properties of the allotypic specificities in the variable region.

This report describes a study of the group a allotypic markers present on homogeneous antibodies. Differences in the ability of these antibodies to absorb antisera against the group a allotypes have been found. These results suggest that each group a allotype finds expression as an array of specificities of which only a limited number are expressed on individual antibody molecules. This situation is in contrast to that described for the light (L) chain allotype b4 (7). Complete b4 specificities are expressed by individual homogeneous antibodies, which suggests that these specificities are located within the constant region of the b4 L chains, an interpretation consistent with the findings of Appella et al. (8) and Frangione and Lamm (9).

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¹ *Abbreviations used in this paper:* Ab, antibody; ECF antiallotype sera, antiallotype sera treated with ethyl chloroformate; H chain, heavy chain; L chain, light chain; PBSA, 0.15 M NaCl buffered to pH 7.9 with 0.02 M phosphate and containing 1% bovine serum albumin; V region, variable region.

Materials and Methods

The preparation of streptococcal vaccines, immunization of rabbits, serologic techniques, and cellulose acetate electrophoresis of sera have been described by Krause (10). Antibodies to Group C streptococcal polysaccharide were isolated on immunoabsorbent columns and further purified by agarose block electrophoresis (11). The antipneumococcal antibodies were a generous gift from Dr. A. D. Strosberg. Disk electrophoresis in urea polyacrylamide gels was performed according to the method of Reisfeld and Small (12). Purified antibody and IgG preparations were radioiodinated by the iodine monochloride method of McFarlane (13) or by the chloramine T method of McConahey and Dixon (14). Antiallotype sera were prepared according to the principles of Oudin (15). Pooled rabbit IgG was isolated by column chromatography on Whatman DE52 DEAE-cellulose in 0.02 M phosphate buffer, pH 7.0, after exhaustive dialysis of the pooled serum against this buffer.

Quantitation of Allotypic Specificity.—The percentage of antibody molecules carrying group a allotypic specificities was determined by radioimmune assay employing antiallotype sera insolubilized by treatment with ethyl chloroformate as described by Avrameas and Ternynck (16). To each of a series of microfuge tubes was added 0.5 μ g of antibody or IgG tagged with ^{125}I in saline buffered with 0.02 M phosphate to pH 7.9 and containing 1% bovine serum albumin (PBSA). This solution also contained sufficient ^{22}Na to provide initially 40,000 cpm to serve as an indicator of residual supernatant (17). Incremental amounts of insolubilized antiserum were added to determine the amount required for maximal binding (Fig. 1), and the total volumes equalized by addition of PBSA as required. After incubation for 2.5 h with gentle agitation, the tubes were centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), most of the supernatant was removed, and the residue counted in a crystal scintillation counter. Each preparation of iodinated material was checked for complete precipitability with insolubilized goat antirabbit Fc- γ and also with 10% trichloroacetic acid.

Inhibition Assays.—By reference to the allotype quantification curves described above an amount of insolubilized antiallotype serum sufficient to bind approximately 40% of the test amount of radioiodinated standard was selected. To this amount of antiserum in a series of microfuge tubes was added incremental (usually doubling) amounts of inhibitor. After mixing, the radiolabeled standard and ^{22}Na were added and the final volumes were equalized with PBSA. After an additional incubation for 2.5 h with gentle agitation, most of the supernatant was aspirated, and the residue counted in a crystal scintillation counter. The percent inhibition, %I, was calculated using the formula

$$\%I = 100 \left[1 - \frac{P_s}{P_o} \right],$$

where P_o is percent of ^{125}I precipitated in the absence of inhibitor, and P_s is the percent precipitated in the presence of inhibitor.

RESULTS

Group a allotypes have antigenic determinants in the variable region of antibody H chains (1-6). Studies were designed to determine whether the expression of the antigenic determinant of a given group a allotype would vary in different antibodies. Initial experiments were carried out with several Group C streptococcal antibodies. Their L chains were homogeneous by several criteria, including disk gel electrophoresis and amino acid sequence at the amino terminus. Homogeneity of the H chains was indicated by allelic

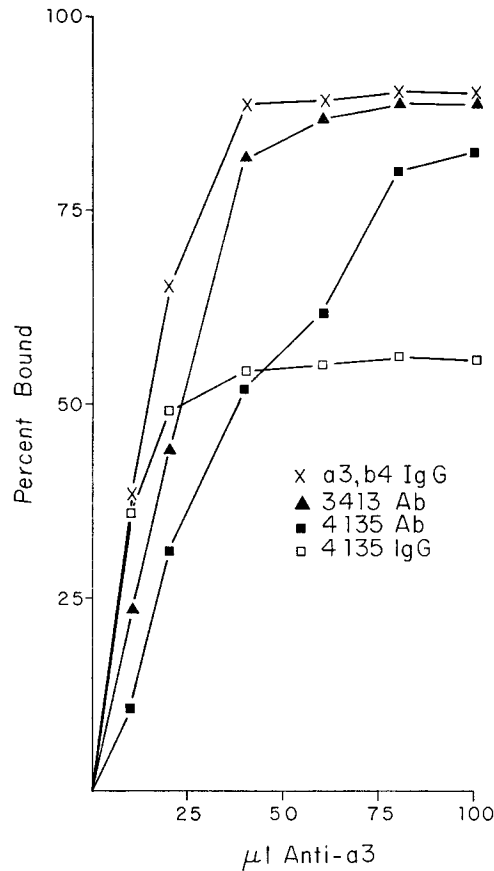


FIG. 1. Binding of radiolabeled a3/b4 IgG pool, a3/b4 3413 antibody, a3/b4 4135 antibody, and preimmune a2,3/b4 4135 IgG. Each point represents 0.5 μg of radiolabeled sample with the designated amount of anti-a3.

exclusion with respect to group a allotypes, as well as by electrophoretic homogeneity of the antibody.

Fig. 1 depicts the binding of radiolabeled a3/b4 antibodies 3413 and 4135, an a3/b4 IgG pool, and preimmune IgG from rabbit 4135, that carried the allotypes a2, 3/b4. Increasing amounts of anti-a3 were added to constant amounts of ^{125}I -labeled samples. The slower rise in the curve for 4135 antibody (Ab) suggests a lesser binding efficiency than that of 3413 Ab. The latter in turn would be somewhat less efficiently bound than the a3/b4 IgG pool. The preimmune IgG from the heterozygous a2, 3/b4 rabbit 4135 binds as efficiently as the a3/b4 pool but levels off at 55%. This is because the IgG in this sample includes molecules of a2 specificity as well as those which carry no detectable group a marker (18, 19).

These differences in the capacities of homogeneous antibodies to bind to the anti-a3 were examined in greater detail. Inhibition of binding experiments utilized unlabeled antibodies and IgG preparations to inhibit the binding of various ^{125}I -labeled preparations to the ECF anti-allotype sera. In Fig. 2 are depicted the results of inhibition of binding of a3/b4 ^{125}I -IgG to ECF anti-a3 by the unlabeled IgG, three homogeneous antibodies, and the preimmune

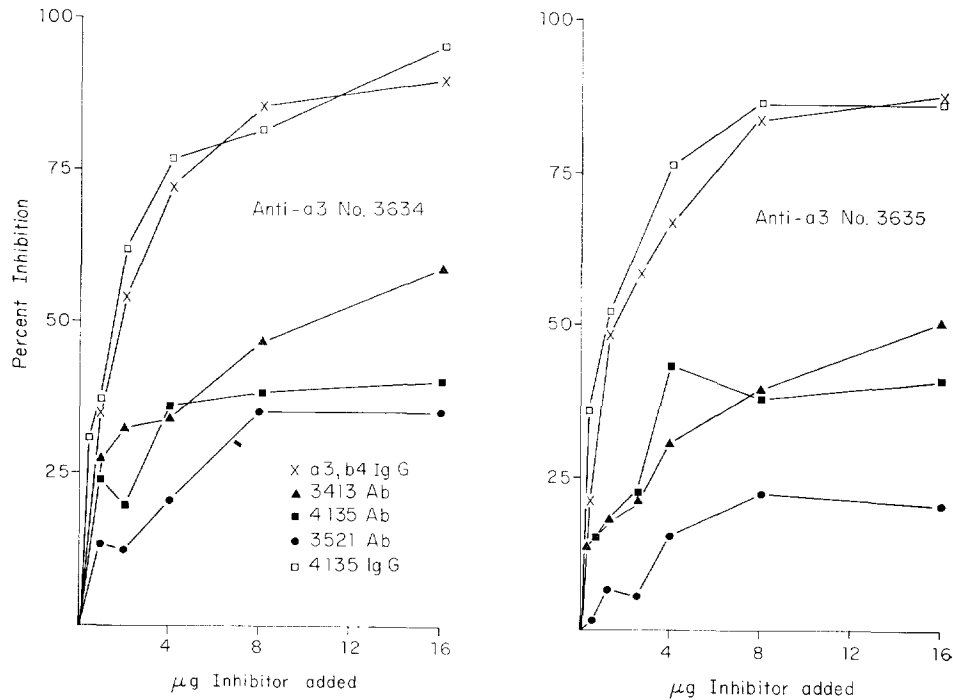


FIG. 2. Left frame: inhibition of binding of radiolabeled a3/b4 IgG pool to ECF anti-a3 by a3/b4 IgG pool, by a3/b4 homogeneous antibodies 3413, 3521, and 4135, and by a2,3/b4 4135 preimmune IgG. The amount of inhibitor added was adjusted to give the indicated amount of allotype-bearing antibody as indicated by radiobinding assay. Right frame: same except a different anti-a3 serum was used.

IgG from one of the rabbits (4135) that produced a homogeneous component used in this experiment. In all the inhibition studies reported in this paper the amount of antibody carrying the cognate allotype was first determined by binding studies such as those presented in Fig. 1. The amount of inhibitor added was then adjusted to provide the indicated amount of allotype-bearing molecules on the basis of the initial binding experiments. This procedure enabled comparisons to be made with antibody preparations containing some molecules either allotypically blank or bearing some allelic allotype.

The inhibition curves in Fig. 2 clearly show that the homogeneous antibodies have an antigenic deficiency compared to the IgG samples. Further, there are

differences in the extent to which the homogeneous antibodies inhibit the a3 IgG binding. It is important to note that, while 4135 Ab does not carry the complete spectrum of a3 antigenic determinants, the IgG from this rabbit does not differ significantly from the pool.

Fig. 2 right gives the data obtained by repeating the experiment of Fig. 2 left using a second anti-a3 produced in a rabbit unrelated to that producing the first anti-a3. The results are similar to those obtained with the first anti-a3 used in the experiment shown in Fig 2 left. While the degrees of inhibition by the homogeneous antibodies vary somewhat, the immunologic deficiency of the homogeneous antibodies is again seen. Similar results were obtained with other anti-a3 sera produced in either The Rockefeller University or City of Hope rabbit colony.

The generality of limited expression of a3 determinants on homogeneous antibodies is evident from the examples presented in Table I. The inhibitors were tested for their ability to inhibit binding of a3 IgG by adding them in 32-fold excess over the radiolabeled IgG to be inhibited. It can be seen in the right hand column of Table I that the IgG preparations gave approximately 90% inhibition of binding regardless of the source. The possibility that the

TABLE I
Inhibition of Binding of a3/b4 ¹²⁵I × IgG to ECF Anti-a3

Rabbit	Allotype of rabbit	Inhibitor preparation*	Allotype of preparation‡	Percent inhibition§
Pool	a1,3/b5	IgG	a1,3/b5	87
BH16301	a3/b5	IgG	a3/b5	91
3500	a3/b4	IgG	a3/b4	92
		Ab-A1	a3/b4	73
		Ab-A2	a3/b4	70
4135	a2,3/b4	IgG	a2,3/b4	87
		Ab-C	a3/b4	40
3521	a2,3/b4	IgG	a2,3/b4	90
		Ab-C	a3/b4	40
3498	a1,3/b4	Antiovalbumin	a1,3/b4	93
3443	a3/b4	Ab-A1	a3/b4	58
		Ab-A2	a3/b4	50
3413	a2,3/b4	Ab-C	a3/b4	60
3582	a3/b4	Ab-A	a3/b4	69
4153	a3/b9	Ab-C	a3/b9	50
2699	a3/b5	Ab-Pn	a3/b5	49
3351	a3/b5	Ab-Pn	a3/b5	85

* The designation A or C after Ab indicated antibodies to components of Group A or C streptococci. Pn indicates antipneumococcal antibody.

‡ All inhibitor preparations were added in 32-fold excess of the radiolabeled a3,b4 IgG. When preparations containing two allotypes were used, a 32-fold excess of a3 IgG was added.

§ The tube containing no inhibitor was 0% inhibition.

|| These antibodies were the kind gift of Dr. A. D. Strosberg. Antibody 2699 is homogeneous whereas antibody 3351 is highly restricted.

effects depicted above arise from selection between allelic a3 variants is unlikely because the IgG from the highly inbred rabbit BH16301 (from the Jackson Laboratory, Bar Harbor, Maine) carries as complete an array of a3 specificities as does the IgG pool. The limited specificities of homogeneous Group A streptococcal antibodies and the pneumococcal antibodies is a further indication of the generality of this phenomenon. All homogeneous a3 Ab thus far tested have expressed an incomplete a3 specificity.

Another question examined was whether or not the specificities on the a3 homogeneous antibodies represent an identical subpopulation. This was investigated by examining the inhibition of binding of homogeneous antibodies with other homogeneous antibodies. Fig. 3 *A* gives the inhibition curves obtained when the binding of ^{125}I 4135 antibody was inhibited with the IgG pool

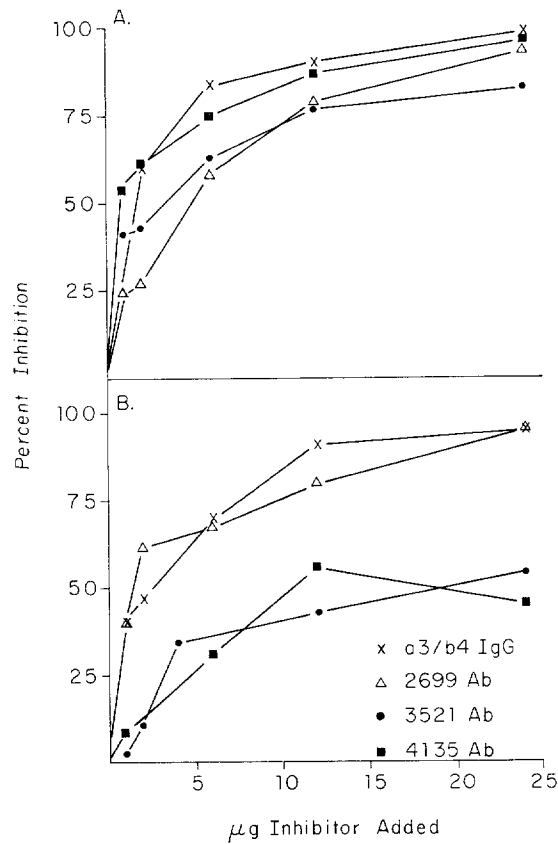


FIG. 3. (A) Inhibition of binding of radiolabeled a3/b4 4135 streptococcal antibody to ECF anti-a3 by a3/b4 IgG pool and homogeneous antibodies a3/b5 2699, a3/b4 3521, and a3/b4 4135. (B) Same as (A) except antibody inhibited was a3/b5 2699 pneumococcal antibody.

and the homogeneous antibodies 2699, 3521, and 4135. While no striking differences are seen in the ability of the antibodies to inhibit this binding, the antibodies 3521 and 2699 were somewhat less effective as inhibitors than 4135 antibody or a3/b4 IgG. Greater differences in inhibition patterns are evident in Fig. 3 B, which presents the inhibition of binding of ^{125}I 2699 pneumococcal antibody to anti-a3 by unlabeled 2699, a3 IgG pool, and by the antibodies 4135 and 3521. The latter two antibodies are much less effective inhibitors than 2699 or a3 IgG pool. The results shown in Fig. 3 suggest that all homogeneous antibodies do not express the same limited a3 specificity, but rather each possesses certain elements of a spectrum of specificities.

It is uncertain how many different specificities there are in this spectrum, but the number may be limited. If this were the case, a small group of a3 homogeneous antibodies should be capable of complete inhibition of all the specificities present in an a3 IgG pool. In Table II are listed the results of an experiment designed to test this postulate. In this experiment, three homogeneous antibodies, alone and in various combinations, were used to inhibit binding of ^{125}I a3/b5 IgG to ECF anti-a3. The data support those presented in Fig. 3. The a3 determinants of 2699 are complementary to those of both 3521 and 4135. It is further evident that 3521 and 4135 antibodies do not equally express a3 markers. In spite of the apparent complementarity of the a3 determinants of these antibodies, all three together still do not express the entire spectrum of the a3 pool.

In additional studies limited expression of the specificities of allotype a1 and a2 have also been observed, although there are not as many examples as for the a3 antibodies. Table III lists inhibition values given for the a2/b4 IgG

TABLE II
*Complementation of a3 Specificities on Homogeneous Antibodies**

Inhibitor	Amount		Inhibition of binding
	$\mu\text{g}\ddagger$	%	
a3/b5 IgG	6	83	
4135 Ab	6	38	
3521 Ab	6	33	
2699 Ab	6	54	
a3/b5 IgG	12	89	
4135 Ab + 3521 Ab	6 + 6	44	
4135 Ab + 2699 Ab	6 + 6	64	
3521 Ab + 2699 Ab	6 + 6	65	
a3/b5 IgG	18	90	
4135 Ab + 3521 Ab + 2699 Ab	6 + 6 + 6	73	

* The inhibitors and the ECF anti-a3 were mixed and then the radiolabeled a3/b5 IgG, 0.5 μg , was added.

‡ The amount of the inhibitor added was adjusted to give the indicated amount of allotype-bearing antibody as indicated by radiobinding assay.

TABLE III
*Inhibition of Binding of ^{125}I a2/b4 IgG to FCF Anti-a2**

Rabbit	Allotype of rabbit	Inhibitor preparation	Allotype of preparation	Percent inhibition
Pool	a2/b4	IgG	a2/b4	85
3664	a2/b4	Ab-C	a2/b4	73
4035	a2/b4	Ab-C	a2/b4	65

* Abbreviations same as designated for Table I.

pool and for two homogeneous a2/b4 antibodies. The differences noted between these antibodies and the a2 IgG pool are significant, although not as great as some of the differences seen between the a3 antibodies and the a3 IgG samples. A similar result was obtained with an a1 pneumococcal antibody obtained from Dr. A. D. Strosberg. These data suggest that a diverse range of specificities are also associated with the other group a allotypes.

DISCUSSION

Antigenic variations in group a allotypic specificities on homogeneous antibodies directed against streptococcal and pneumococcal polysaccharides have been observed. The homogeneous antibodies tested expressed only a portion of the specificities exhibited by the total IgG of the rabbit even though these antibodies reacted completely with group a antisera. This latter property distinguishes these antibodies with variations in group a allotypic specificity from those antibodies which lack completely a group a allotype (18, 19).

An explanation of the diverse expression of group a allotypes based on the assumption of allelic subpopulations is rendered unlikely by the finding that all total IgG preparations from individual rabbits expressed the complete specificity of the appropriate allotype. These samples included IgG from group a heterozygous individuals as well as IgG from a highly inbred rabbit from the Jackson Laboratory colony at Bar Harbor. That some specificities are common to individual antibodies was indicated by the experiments comparing homogeneous antibodies to one another (Fig. 3).

The range of specificities in the a3 pool was only partially reconstituted by a mixture of three different homogeneous antibodies. This limited reconstitution may stem from the fact that the homogeneous antibodies were themselves directed against the narrow range of antigenic specificities exhibited by the bacterial polysaccharides. Thus we believe that there is a basic conformation characteristic of each allotype upon which the sequence changes responsible for the antibody combining site impose modifications. Indeed, combination of an antibody with its antigen may produce further modifications of an allosteric nature, although such an effect was not observed in the studies of Spring et al. (20). While we have no way of estimating the precise number of specificities that may exist, the number must be constrained by the necessity of avoiding cross-reaction with the specificities arising from other group a allo-

types and by the fact that within a given allotype there will be cross-reacting specificities. The summation of all of these effects would be expected to elicit in individual antibodies a range of specificities which in some cases might approach the total expressed in the IgG pool. Because complete homogeneity tests must ultimately rest on sequence studies, it is not possible to distinguish between complementation arising from partial heterogeneity in the antibodies tested and a high degree of expression of the range of allotypic specificities which in principle could be exhibited by a completely homogeneous antibody. Our data show that antibodies directed against polyantigenic substances, such as ovalbumin, exhibit a complete range of specificities (Table I). In addition, the studies of Spring et al. (20) indicate that purified antibody populations directed against haptenic antigens express the complete range of allotypic specificities. None the less, it is clear from the studies reported here that individual antibodies can be deficient with respect to the total range characteristic of an IgG pool.

In contrast to the findings reported here with the group a allotypes, a previous study (7) indicated that homogeneous L chains carrying b4 allotypes were serologically indistinguishable even though these L chains showed considerable variation in their amino terminal sequences. This may be a reflection of the different locations of the group a and b allotype determinants within the variable region of the H chains and the constant region of the L chains, respectively. Even though V_L region correlates have been observed (21, 22) for the group b allotypes, the determinants are most likely in the constant (C_L) region (8, 9). The group a determinants are most likely located in the V_H region (1-6), which places them in close proximity to the binding site determinants. Thus it is not unexpected that differences in binding sites would cause variable expression of allotypic determinants.

In spite of this proximity, the binding site may not participate directly in the group a determinant. Indeed, Spring et al. (20) showed that hapten antigens did not inhibit binding of anti-allotype sera, whereas a similar study utilizing anti-idiotypic sera (23) showed that the hapten would inhibit this binding. A more recent study (24) indicated that there is not an obligatory association between idiotypic and group a allotypic determinants. Thus the amino acid determinants of the group a specificities are probably distinct from the determinants of the binding site. The phenomenon observed here is more likely due to conformational changes imposed by binding site differences. Alternatively, it may reflect amino acid sequence variations in V_H regions other than the binding site. If the latter interpretation is true, comparison of homogeneous antibody sequences to locate the amino acid residues which determine the specificity of the allotypes will be complicated by these antigenic variants.

SUMMARY

Variations in the ability of homogeneous antibodies to absorb group a allotype antisera suggest that these V_H region allotypes comprise a spectrum

of specificities. While no homogeneous antibody tested would completely absorb antisera to the group a specificities, the total IgG preparations from individual rabbits was capable of doing so, including preimmune IgG from the rabbits that produced the homogeneous antibodies.

Differences in the serologic reactivities of homogeneous antibodies indicate that each possesses only a portion of the spectrum of the allotypic specificities expressed by the total IgG.

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