Association of Allotypic Specificities of Group a with Allotypic Specificities A11 and A12 in Rabbit Immunoglobulin*

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ABSTRACT: Immunoglobulin G (IgG) from rabbits heterozygous with respect to the heavy (H) chain markers of both the group a allotypes and the A11 and A12 allotypes has been cleaved by treatment with cyanogen bromide in hydrochloric acid.

The nature of the cleavage products is determined by the A11 and A12 determinants. The products have been separated by gel filtration employing Sephadex G-200. The group a specificities present on the fragments have been determined by their ability to inhibit the precipitation of radioiodinated IgG by homologous group a antiallotype serum. The results show that the H chains of the IgG molecules bear the group a allotypic specificity inherited from the same parent from which the A11 or A12 specificity was inherited. Thus, if separate genes controlling the allotypic specificities identified on different regions of the H chains are involved in the synthesis of a single chain, both genes must be on the chromosome of one parent.

Allotypic markers (Oudin, 1956) have now been recognized on the immunoglobulin molecules of a number of animal species.[1] The distribution of the allotypic markers on the various heavy (H) chains[2] characterizing each of the immunoglobulin classes of the rabbit has presented an enigma with respect to the genetic control mechanisms. The allotypic specificities of group a (a1, a2, and a3) are present on the H chains of IgG (γ chain) (Stemke, 1964), IgM (μ chain) (Todd, 1963), IgA (α chain) (Feinstein, 1963), and IgE (ϵ chain) (Kindt and Todd, 1969). These specificities are associated with characteristic changes in the amino acid compositions of peptides from the N-terminal region of γ chains (Koshland, 1967; Koshland *et al.*, 1968; Prahl and Porter, 1968), α chains (Wilkinson, 1969b), and μ chains (Koshland *et al.*, 1969). Recently Wilkinson (1969a) has related these differences directly to amino acid sequence variations for the a1 and a3 specificities. Each of the H chains (γ, α, μ, and ϵ), which are specific for the immunoglobulin classes, is distinguished by characteristic Fc regions. Thus each class of H chain seems to participate in a common genetic polymorphism in its amino-terminal region, while retaining a constant individuality in its carboxy-terminal region.

One mechanism by which this could occur would require that two distinct genes participate in the synthesis of a single H chain. Thus a series of class specific genes coding for the constant region, including approximately three-quarters of the H chain to the carboxy terminus, might be visualized. Each of these genes might then interact in some way with a common gene coding for at least that part of the remaining portion of the H chain near the amino terminus which codes for the allotypic specificity.[3] Similar mechanisms have been proposed as solutions to similar problems in the genetic control of light (L) chain synthesis (Dreyer and Bennett, 1965; Hood and Ein, 1968).

Should the genetic control of H chain biosynthesis involve the interaction of two genes or their messages, it might be possible that this mechanism would allow one gene to be inherited from one parent and the second gene from the other. If a major portion of the H chains within individual animals was regularly found to share genetic determinants from both parents, it would seem clear that two genes must routinely interact in the synthesis of a single chain (Prahl *et al.*, 1969a, and Dubiski, 1969a,b).

A demonstration of such interaction would require a way of identifying the contribution of each gene from each parent. The group a allotypic markers are available to identify the contribution of the hypothetical common gene for the amino-terminal region. Two new allotypic markers on the γ chain, A11 (Mandy and Todd, 1968, 1969) and A12 (Mandy and Todd, 1970), are now available as markers for the class specific gene of IgG. The A11 marker has been related to a methionine occupying a position which is adjacent and amino terminal to the cysteine which participated in the inter-H-chain disulfide bond in the hinge region of the γ chain. In molecules possessing the A12 specificity, this position is occupied by threonine (Prahl *et al.*, 1969a,b).

Reaction with cyanogen bromide (CNBr) has been shown to cleave peptide chains on the carboxyl side of methionine (Gross and Witkop, 1962). For the study reported here, it is particularly fortunate that one of the markers involves a

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[1] For a summary of this subject, see reviews by Oudin (1966) and Kelus and Gell (1967).

[2] The nomenclature employed for rabbit immunoglobulins, their chains, and fragments follows that recommended by a committee of the World Health Organization (1964).

[3] This problem has been discussed in greater detail in Prahl *et al.* (1969a).

methionine on the amino side of the inter-H-chain disulfide bond. It has been shown that treatment of A12 IgG with CNBr in 0.3 N HCl cleaves it to a 5S fragment, whereas similar treatment of A11 IgG produces an additional cleavage at the methionine adjacent to the cysteine participating in the inter-H-chain disulfide bond to yield 3.5S fragments (Kindt *et al.*, 1970). IgG from rabbits heterozygous with respect to A11 and A12 is cleaved to yield 5S and 3.5S fragments. The fragments can be separated by gel filtration. These two types of cleavage, which are basic to this paper, are depicted schematically in Figure 1.

By use of this difference in cleavage by CNBr together with Sephadex gel filtration, the IgG molecules bearing the group a allotypic specificities can be separated into two populations based on whether or not the H chains bear the methionine characteristic of A11. These procedures permit one to examine the possibility of two genes, one from each parent, interacting to form a single γ chain.

Materials and Methods

Serologic Methods. Typing of sera for the allotypic specificities of groups a and b was done by interfacial precipitation reactions (ring tests). The antiallotype sera were prepared by injection of rabbits with rabbit antiovalbumin specific precipitate (Oudin, 1960). Typing for A11 and A12 was done by inhibition of hemagglutination (Mandy and Todd, 1969, 1970).

Isolation of IgG. IgG was obtained from the sera of individual rabbits by two precipitations with 1.75 M $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose (Levy and Sober, 1960).

Radioiodination. IgG was tagged with ^{125}I using the iodine monochloride method (McFarlane, 1958).

Cyanogen Bromide Cleavage. The IgG (10 mg/ml) was dissolved in pH 6.5 0.0175 M sodium phosphate buffer, and an equal volume of CNBr (15 mg/ml) in 0.6 N HCl was added (Cahnmann *et al.*, 1966). The reaction mixture was allowed to stand at room temperature for at least 4 hr and then dialyzed against 0.1 M potassium phosphate buffer, pH 6.8. Under these conditions recoveries of protein as determined by OD_{280} were found to be better than 75%. This reaction as it pertains to A11 and A12 IgG is discussed in greater detail by Kindt *et al.* (1970).

Gel Filtration. Gel filtration was performed at room temperature with a column (90 × 2.5 cm) of Sephadex G-200 in pH 6.8 0.1 M potassium phosphate buffer containing 0.03% sodium azide.

Measurement of Allotypic Specificity in IgG Fragments. The allotypic specificities of the fragments obtained by cleavage with CNBr were determined by the ability of the fragments to block precipitation of radioiodinated IgG by homologous antiallotype serum. The fragments (0–50 μg) in pH 6.8 phosphate-buffered saline (PBS) were added to 0.1-ml portions of antiallotype serum in Beckman Microfuge tubes. The volume in each tube was adjusted to 0.25 ml with PBS, and the tubes were incubated for 2 hr at 37°. IgG labeled with ^{125}I (35 μg) was added to each tube. The tubes were incubated an additional 2 hr at 37° and overnight at 4°. The tubes were spun 5 min in the Microfuge. The supernatant solutions were discarded, and the precipitates were washed two times with PBS. The radioactivity of the precipitates

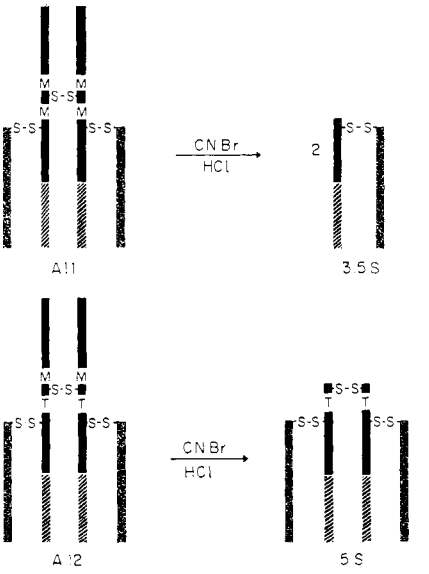

FIGURE 1: The action of CNBr on IgG molecules bearing the allotypic determinants A11 and A12. M represents methionine and T represents threonine. The diagonally cross-hatched areas represent the regions in which the group a determinants reside.

was determined directly by placing the Microfuge tube in a counting tube and counting in a crystal scintillation counter. Results are reported as the per cent of counts recovered in the precipitates of control tubes to which no inhibitor protein was added. All data were calculated from a minimum of 12,000 accumulated counts.

Results

In a previous study of the cleavage of A11 and A12 IgG molecules by CNBr (Kindt *et al.*, 1970) it was shown that A12 IgG yielded predominantly 5S fragments in agreement with the results of Cahnman *et al.* (1966) (Figure 1). Although 3.5S fragments were obtained from A11 molecules, a small but significant portion of the IgG molecules from an A11 rabbit yielded 5S fragments. The 5S fragments appear to come from a subpopulation of IgG behaving differently on treatment with CNBr, rather than simply unreacted molecules, since isolation of the 5S fraction and retreatment with CNBr did not lead to further cleavage. Thus the 3.5S pool following Sephadex G-200 filtration would contain fragments only from the A11 molecules, whereas the 5S pool would contain fragments from A12 and, to a lesser extent, also A11 molecules.

Figure 2 presents the results of a control experiment in which IgG isolated from an a1,A12 rabbit is mixed with IgG isolated from an a3,A11 rabbit.[4] The light-chain allotype, b4, was the same in both rabbits and is not of concern here. This mixture was treated with CNBr in 0.3 N HCl and then subjected to Sephadex G-200 gel filtration. Fragments eluting

[4] In considering the data of Figures 2 and 4 it should be recognized that the slopes from the 5S and 3.5S fragments relative to one another for a given antiserum are the important considerations. To compare the slope of a line from the anti-a1 serum with the slope of a line from the anti-a3 involves a comparison of the relative strengths of the two antisera. Such a comparison is not pertinent here.

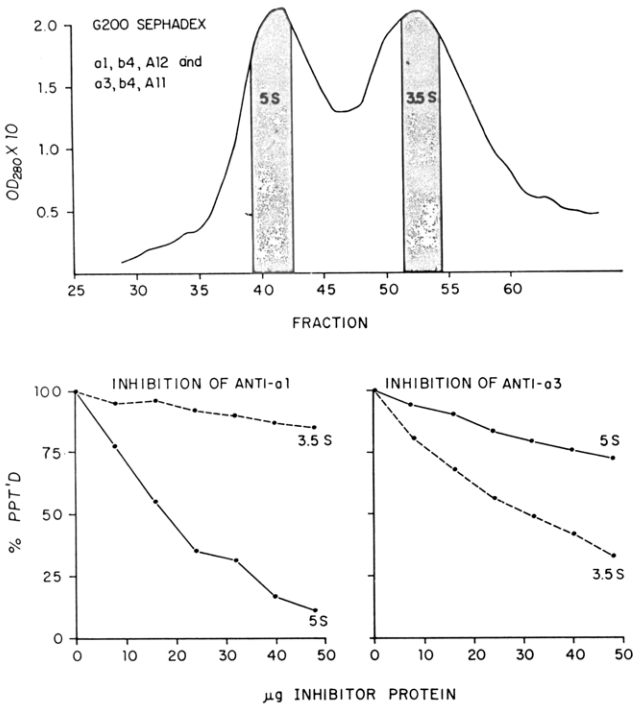

FIGURE 2: Upper panel: a CNBr digest of a mixture of 18 mg each of IgG from rabbit 1196 (a1, b4, A12) and from rabbit 1289 (a3, b4, A11) was passed through a column (90 × 2.5 cm) of Sephadex G-200. Fractions of 6.6 ml were collected. The fractions of each shaded area were pooled and concentrated by pervaporation. Lower left panel: inhibition of precipitation of 35 µg of a3,b4,A12 IgG · ^{125}I with 0.1 ml of anti-a1 by the fragments of the two peaks. Lower right panel: inhibition of precipitation of 35 µg of a3,b4,A12 IgG · ^{125}I with 0.1 ml of anti-a3. The percentage precipitated is relative to the number of ^{125}I counts precipitated in the absence of inhibitor.

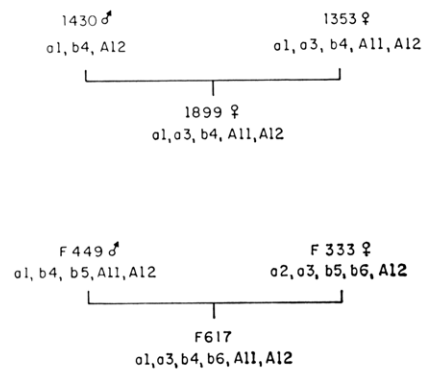

FIGURE 3: Breeding pedigrees for rabbits 1899 and F617. Because of the linkage observed in the inheritance of the group a and the A11 and A12 allotypes, it could be concluded that rabbit 1899 had inherited a1 and A12 from the father and a3 and A11 from the mother. Contrariwise, rabbit F617 would have inherited a1 and A11 from the mother and a3 and A12 from the father.

characteristically as 5S and 3.5S molecules were obtained (upper panel). The lower panels present the results of experiments in which the 5S and 3.5S fragments were examined for their ability to block precipitation of radioiodinated a1 and a3 IgG by homologous antiserum. It can be seen that material from the 5S peak is very effective in blocking anti-a1, as expected from the composition of the starting mixture. An equivalent amount of blocking protein would be 24 µg. Slight blocking of a1 precipitation is observed for the 3.5S material. This may reflect a low level of contamination of the 3.5S peak with material from the 5S peak due to imperfect resolution in the gel filtration. The results with anti-a3, as expected, show that the material of the 3.5S peak is very effective in blocking anti-a3. The material from the 5S peak exhibited moderate blocking ability with anti-a3. The degree of blocking by the 5S peak has always been found greater than expected from the genetic origins of the IgG used. In part this may represent a low level of contamination of the 5S peak with material from the 3.5S peak, but the principal cause is believed to be the presence of 5S fragments previously shown to be present in the cleavage product from A11 IgG.

For this reason it was considered desirable to do the experiment with the A11 marker associated with first one and then the other of the group a specificities. Two possibilities exist for the genetic makeup of a doubly heterozygous a1,a3, A11,A12 rabbit. In one of these a1 and A12 are inherited from one parent and a3 and A11 from the other parent. In this case, a1 and A12, or a3 and A11, would normally be transmitted together to the progeny of such a rabbit because of the genetic linkage which exists (Zullo *et al.*, 1968). In the second possibility, a1 and A11 are inherited from one parent, and a3 and A12 are inherited from the other. In this case, a1 and A11 or a3 and A12 would normally be transmitted together to the progeny.

TABLE 1: Inhibition of Group a Antiallotype Sera by CNBr Fragments.

	Per Cent Inhibition[a] of Antiserum					
	a1		a2		a3	
Rabbit Allotype	3.5 S	5 S	3.5 S	5 S	3.5 S	5 S
1289 + 1196 a3,b4,A11 + a1,b4,A12	8	65			44	18
1899 a1,a3,b4 A11,A12	4	64			46	17
F617 a1,a3,b4, b6,A11,A12	78	34			3	33
2515 a1,a2,b4, A11,A12	85	71	1	58		
1218 a2,a3,b9, A11,A12			3	60	77	67
232 a2,b4,b5, A11,A12			51	54		
1289 a3,b4,A11					48	42

[a] Inhibition of radioprecipitation by an equivalent amount of the given fragment.

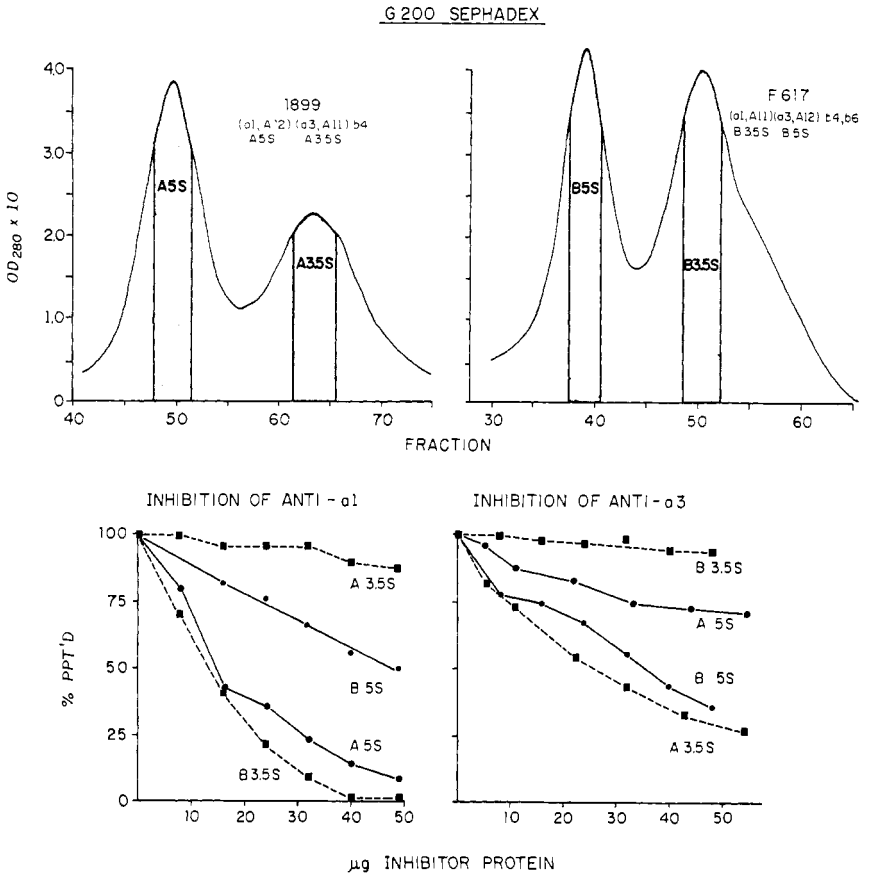

FIGURE 4: Upper panels: Sephadex G-200 filtrations through a 90 × 2.5 cm column of CNBr digests of IgG. Fractions of 5.3 ml (left panel) and 6.9 ml (right panel) were collected. The fractions of each shaded area were pooled and concentrated by pervaporation. The H-chain allotypes inherited from each parent are enclosed in parentheses. The major peak to which these allotypic combinations contribute is indicated below the parenthesized specificities. Lower left: inhibition of precipitation of 35 μg of a1,b4,A12 IgG·^{125}I by 0.1 ml of anti-a1. Lower right: inhibition of precipitation of 35 μg of a3,b4,A12 IgG·^{125}I by 0.1 ml of anti-a3. The per cent precipitated is relative to the number of ^{125}I counts precipitated in the absence of inhibitor.

Rabbits of both genetic types were selected for these experiments as shown by their pedigrees (Figure 3). Rabbit 1899 inherited a1 and A12 from one parent and a3 and A11 from the other. Rabbit F617 was the reverse; it inherited a1 and A11 from one parent and a3 and A12 from the other. Despite the genetic origins of the markers, it is conceivable that in rabbit 1899 the hypothetical A11 gene might react with the hypothetical a1 gene to give a1,A11 γ chain. In this case fragments bearing the a1 specificity should be found in the 3.5S pool following cleavage with CNBr. For rabbit F617 the reverse possibility should be considered. The 3.5S pool might contain fragments bearing the a3 specificity.

The results obtained with the IgG samples isolated from these two rabbits are presented in Figure 4. Although both samples of IgG were obtained from doubly heterozygous rabbits, their relative contributions to the two peaks are not equal. Several studies have shown that the degree with which various allotypic markers find expression is variable (Oudin, 1966). It is seen that the slope for the 3.5S fragments from rabbit 1899 (designated A) with anti-a1 is essentially that seen in the control experiment (Figure 2), *i.e.*, few, if any, 3.5S fragments bearing the a1 allotypic specificity were present. The slope for the 3.5S fragments from rabbit F617 (designated B) with anti-a3 was essentially similar, *i.e.*, few, if any, 3.5S fragments bearing the a3 allotypic specificity were present. The slope for the 5S fragments from F617 showed moderate blocking activity against anti-a1, and the 5S fragments from 1899 showed moderate blocking activity against anti-a3 similar to that attributed in the initial control experiment to the IgG subpopulation in the A11 rabbits resistant to cleavage by CNBr to 3.5S fragments. In the absence of further knowledge concerning the nature of this population of molecules, particularly its variation in concentration from rabbit to rabbit, no conclusion can be drawn from these slopes except that they certainly represent less blocking ability than is demonstrated by the 5S fragments from the rabbits in which A12 is genetically linked, respectively, to the a1 and a3 specificities. These 5S fragments were essentially as effective as their 3.5S counterparts (3.5S from F617 against anti-a1 and 3.5S from 1899 against anti-a3) in blocking the precipitation of radioiodinated IgG by homologous antiserum.

The data from the experiments depicted in Figures 2 and 4 are summarized in Table I together with similar results obtained with IgG from other rabbits. For rabbit 2515, it is clear that the A11 is not associated with a2, since only 1% inhibition of anti-a2 was obtained with the 3.5S fragment. The considerable blocking of anti-a1 by the 5S fragments is

attributed to A11 molecules not cleaved by CNBr to 3.5S fragments. Again for rabbit 1218, it is clear that A11 is not associated with a2, since only 3% inhibition of anti-a2 was obtained with 3.5S fragments. Again the contribution of the A11 molecules resistant to cleavage to 3.5S fragments by CNBr is seen in the 67% blocking of anti-a3 by the 5S fragment. Rabbit 232, homozygous for a2 and heterozygous for A11 and A12, yielded 3.5S and 5S fragments essentially equivalent in their ability to block anti-a2. This result demonstrates that a2 and A11 can coexist on the same chain. As expected from the initial control experiment employing a mixture of A11 and A12 IgG, rabbit 1289, homozygous for both a3 and A11, gave 3.5S and 5S fragments equally effective in blocking anti-a3. These results, as summarized in Table I, lead us to the conclusion that we would have detected gene interaction were it responsible for as much as 5% of the product.

Discussion

The results reported here demonstrate that nearly all, if not all, γ chains bearing both group a and A11 or A12 allotypic markers preserve at the molecular level the association determined by the genetic linkage through which they were inherited. Thus interaction, either direct or indirect, of a gene for a group a allotypic determinant present on the chromosome from one parent with a gene for the C-terminal constant region, present on the chromosome from the other parent, does not play a major role, if any, in the synthesis of γ chains.

The findings in the present paper are in agreement with those of Hamers and Hamers-Casterman (1967) with respect to allotype A8. This specificity is found in the Fc region of IgG molecules from some rabbits possessing allotype a1. Ultracentrifugation experiments with complexes formed by the interaction of anti-A8 serum and radioiodinated IgG from an a1,a2,b4,b5,A8 rabbit lead to the conclusion that the radioiodinated IgG in the complex was primarily a1, with little or no a2 specificity being present.

The conclusion that genes on the maternal and paternal chromosomes cannot interact within the cell to form a single γ chain carries the tacit assumption that both are present. Somatic segregation of maternal and paternal chromosomes has been shown to occur in the deer mouse. This segregation has been suggested as a possible explanation for the apparent hemizygous expression observed in immunoglobulin synthesis (Ohno *et al.*, 1966). If chromosomes from both parents are not present within the cells producing immunoglobulins, it would not be surprising if products coded by both genes were absent.

It should be emphasized that the results reported here in no way suggest that two genes present on the same chromosome do not interact in the synthesis of a single γ chain. Since the γ chains appear to be synthesized with a single growing point (Fleischman, 1967; Knopf *et al.*, 1967), the contributions of the two genes, if interaction occurs, must occur prior to the translation of the message. There would seem to be an advantage to both genes being present on the same chromosome. Presumably they might more readily find one another to interact either directly (Smithies, 1967) or indirectly by a copy choice mechanism in transcription to messenger RNA. If interaction occurs at the message level, the activation of a gene on one chromosome must preclude the activation of the second gene on the other chromosome. Rigorous selectivity in gene activation seems to be the rule in immunoglobulin synthesis.

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