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# A New Allotypic Specificity Found on Rabbit Aa Locus Negative IgG-immunoglobulins

Michael I. Luster

*Loyola University Chicago*

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A NEW ALLOTYPIC SPECIFICITY FOUND ON  
RABBIT Aa LOCUS NEGATIVE IgG-IMMUNOGLOBULINS

by  
Michael I. Luster

A Thesis Submitted to the Faculty of the Graduate School of  
Loyola University (Chicago) in Partial Fulfillment of  
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LOYOLA UNIVERSITY MEDICAL CENTER

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## INTRODUCTION

The genetic heterogeneity of IgG-immunoglobulins is presently being studied intensively. The goal of this research is to link the antigenic differences located on immunoglobulin molecules to variations on the chromosome itself. Thus, the final objective would be to utilize these antigenic determinants as markers on the chromosome in elucidating the synthesis and control of antibody formation.

The genetic heterogeneity of IgG-immunoglobulin molecules distinguishes several types of antigenic specificities: a) the isotypic specificities are those which are uniform among individuals of one animal species (11, 39). These isotypic specificities make it possible to distinguish five classes (IgG, IgA, IgM, IgD and IgE) of immunoglobulins, four subclasses of IgG ( $\gamma_1$ ,  $\gamma_2$ ,  $\gamma_3$  and  $\gamma_4$ ), at least two subclasses of IgA, two probable subclasses of IgM and two types (Kappa and Lambda) of immunoglobulin light chains (6); b) the allotypic specificities are those which, being found on molecules of identical isotypic specificities, are different for individuals within the same species (6, 43, 44); c) the idiotypic specificities, which are located on the Fab portion of IgG molecules, designate antigenic specificities of immunoglobulins which are peculiar in two respects. First, each of them is peculiar to antibodies against one given antigen, and second, each is peculiar to one individual animal species producing

those antibodies (18, 19, 30, 46).

Schultze (50) reported the first isoimmunization with serum proteins in 1902. In this study, subcutaneous injection of whole rabbit serum into another rabbit resulted in the production of isoprecipitins which reacted with the serum of some but not all rabbits. Schultze's observations were not extended until Oudin (43) in 1956 isoimmunized rabbits with purified immunoglobulins. The immunized rabbits produced isoprecipitins which were utilized to detect genetic variations between individual rabbits. A total of four specific precipitating antisera, distinguished by their reactions with some, but not all rabbit sera, were produced. Oudin termed the immunogenic determinants allotypes, and the phenomenon allotypy (43). Since Oudin's report, allotypy has been observed on rabbit  $\alpha$ 2-macroglobulin (4, 27), haptoglobulin (8), IgM (25), and IgA (9, 33) immunoglobulins, low density lipoproteins (1, 2) and  $\alpha$ 1-aryl esterase (29).

In 1958 Dray and Young (11) reported that isoimmunization with whole rabbit serum produced antisera which gave precipitin bands in the  $\alpha$ ,  $\beta$  and  $\gamma$  regions upon electrophoresis. The results of Oudin (43) and Dray and Young (11) were confirmed by Dubiski et al. (14), by immunizing rabbits with bacteria agglutinated with rabbit antisera. Immuno-electrophoresis experiments indicated that the anti-allotype antisera of Dubiski were reacting with the IgG-immunoglobulins present in rabbit sera (14).



## IDENTIFICATION OF RABBIT ALLOTYPES

In 1960 Oudin (44, 45) reported the presence of six anti-allotype antisera in rabbits. Genetic analysis indicated that the six allotypes are controlled by two series of three allelic genes at two unlinked loci. The allelic genes of the a locus, designated a<sup>1</sup>, a<sup>2</sup> and a<sup>3</sup>, control the allotypic specificities a<sub>1</sub>, a<sub>2</sub> and a<sub>3</sub> of rabbit IgG-immunoglobulin heavy chains<sup>1</sup>. The allelic genes of the b locus designated b<sup>4</sup>, b<sup>5</sup> and b<sup>6</sup>, control the allotypic specificities b<sub>4</sub>, b<sub>5</sub> and b<sub>6</sub> of rabbit light chains. The b<sub>5</sub> and b<sub>6</sub> allotypes have determinants in common, but the two allotypes are distinct (25). The a and b loci are not closely linked to each other or to the sex chromosomes (25). Later, Dubiski and Muller (15) identified an allotype designated b<sub>9</sub>, controlled by the b<sup>9</sup> gene, which was found to segregate as an allele at the b locus.

Analysis with the seven anti-allotype antisera of fragments from rabbit IgG-immunoglobulin molecules prepared by papain digestion indicated that the a and b loci allotypic determinants are associated with the Fab fragment and not the Fc fragment (13). The a locus specificities are associated with the Fd portion of the heavy chains (17), and the b locus specificities are associated with the light chains (17, 51, 57).

<sup>1</sup> Allotypic specificities Aa<sub>1</sub>, Aa<sub>2</sub>, Aa<sub>3</sub>, Ab<sub>4</sub>, Ab<sub>5</sub>, Ab<sub>6</sub>, Ab<sub>9</sub>, Ac<sub>7</sub> and Ac<sub>21</sub> are abbreviated to a<sub>1</sub>, a<sub>2</sub>, a<sub>3</sub>, b<sub>4</sub>, b<sub>5</sub>, b<sub>6</sub>, b<sub>9</sub>, c<sub>7</sub> and c<sub>21</sub>; the genes are a<sup>1</sup>, a<sup>2</sup>, a<sup>3</sup>, b<sup>4</sup>, b<sup>5</sup>, b<sup>6</sup>, b<sup>7</sup>, c<sup>7</sup> and c<sup>21</sup>.

Hamers et al. (22, 23, 24) observed that some rabbit antisera produced two precipitin bands, designated anti-al' and anti-al", when reacted with serum from al animals, while others produced only one band identical to the al' precipitin band. Progeny data indicated that a new antigenic specificity, designated a8, under the control of a gene closely linked to the al gene was represented by the second band. Monospecific anti-a8 was obtained by absorbing anti-al" with rabbit sera that gave only the single (al') precipitin band. The a8 allotypic determinant is located on the Fab fragment of IgG-immunoglobulin molecules.

Mandy and Todd (36, 37, 38) utilized agglutination-inhibition techniques in detecting two allotypic determinants designated A11 and A12. Data from breeding studies indicated that the A11 and A12 allotypes represent a group of heavy chain specificities distinct from the a locus allotypic specificities. Immunochemical studies (26, 37) and amino acid sequence studies (49) indicated that the A11 and A12 allelic determinants are carried in the hinge region of IgG-immunoglobulin molecules.

Dubiski et al. (13, 31) have described two allotypic determinants designated A14 and A15, located on the Fc fragment of IgG-immunoglobulin molecules. The antisera against the A14 and A15 allelic markers are non-precipitating. The A14 and A15 allotypic specificities appear to be genetically linked to the specificities of the a locus. The synthesis of these two

alleles has been assigned to an additional locus which is referred to as the e locus and the corresponding genes are referred to as e<sup>14</sup> and e<sup>15</sup>.

Two allotypic determinants designated c7 (21) and c21 (35) have been located on the light chains of some rabbit IgG-immunoglobulins. Since the c7 and c21 specificities are found in rabbits heterozygous at both the a and b loci, their synthesis appears to originate from a third locus, designated the c locus. The c7 and c21 specificities appear to be inherited as codominant alleles at the c locus. However, in some animals the c7 and c21 specificities appear to be inherited as a phenogroup suggesting that they may be controlled by genes which are closely linked (21, 35). The c locus is not linked to the a or b loci (21).

#### IMMUNOGLOBULINS LACKING a OR b LOCUS SPECIFICITIES

In normal rabbits, approximately 5% to 30% of the IgG-immunoglobulin molecules lack the a and/or b locus specificities and are referred to as a-negative a(-) and/or b-negative b(-), respectively (5, 20, 32, 47). These molecules appear to be controlled by genes at a locus other than the a or b loci, since they are found in animals that are heterozygous at the a and b loci (20). C- and N- terminal amino acid sequence studies by Apella et al. (3) indicated that the b(-) light chains isolated from a suppressed rabbit (see Allotype Suppression) represent a class of rabbit lambda light chains, while the b(+) light chains apparently represent a class of rabbit kappa light chains.

In analagous experiments, Vice et al. (53) have isolated a locus negative heavy chains from an a locus suppressed rabbit. The a(+) and a(-) IgG-immunoglobulins appear to represent subclasses of the heavy chains.

### ALLOTYPE SUPPRESSION

#### Allotype suppression in heterozygotes

A persistant alteration in the quantitative expression of the a or b allelic allotypes occurs in heterozygous rabbits exposed during fetal and neonatal life to antibody specific for the allotype inherited from the gene of the father.

"Allotype suppression" can be effected either by uterine transfer of anti-allotype antibody or by injection of newborn heterozygous rabbits with anti-allotype antisera directed against the paternal allotype (10, 34). For example, when b<sup>4</sup>b<sup>4</sup> heterozygous mothers are immunized against the paternal b<sup>5</sup> allotype, the b<sup>4</sup>b<sup>5</sup> heterozygous offspring throughout their lives have very low levels of serum b<sup>5</sup> allotype and compensatory levels of the b<sup>4</sup> allotype (10, 34).

#### Suppression of homozygotes

Dubiski (12) performed the first successful homozygous allotype suppression. In this technique b<sup>5</sup>-suppressed b<sup>4</sup>b<sup>5</sup> heterozygous mothers were mated to b<sup>5</sup>b<sup>5</sup> homozygous bucks. Since the b<sup>5</sup>b<sup>5</sup> offspring had no detectable b<sup>5</sup> allotype present at birth from maternal transfer, the injected anti-b<sup>5</sup> was free to react on the cell population. The total IgG levels in the sera of the b<sup>5</sup>-suppressed b<sup>5</sup>b<sup>5</sup> animals were normal, suggesting a

compensatory increased production of b locus negative light chains. However, several problems were associated with this procedure. First, half the offspring were b5b5 homozygotes and half were b4b5 heterozygotes, making it difficult to distinguish the genotype of the newborn. Secondly, b5-suppressed b4b5 rabbits may still produce small quantities of the b5 light chains which might interfere with the full effectiveness of the injected anti-b5.

Vice et al. (54, 56) utilized a zygote transfer technique to alleviate the problem of maternal transfer of IgG-immunoglobulin molecules possessing the allotypic determinant which is to be suppressed. In this procedure, the b5b5 homozygous donor was mated to a b5b5 buck. Twenty four to thirty six hours later the b5b5 zygotes were transferred to the uteri of artificially ovulated b4b4 mothers. At birth, the b5b5 offspring possess only b4 IgG-immunoglobulin molecules as a result of uterine transfer. Injection of anti-b5 antiserum into these b5b5 offspring from foster mothers resulted in the production of animals whose IgG-immunoglobulin molecules were essentially all b locus negative from birth to at least 7 months of age (55).

The b(-) IgG molecules were found to have allotypic specificities c7 and/or c21 of the c locus. In additional experiments by Vice et al., (54) utilizing partially b5-suppressed b5c7c21 rabbits, essentially 100% of the IgG-immunoglobulin molecules were shown to possess the b5, c7 or c21 specificities. However, in partially b5-suppressed b5c7 or

b5c21 homozygous animals only 50% of the molecules possessed the b5, c7 or c21 specificities indicating the existence of an additional undetermined allotypic determinant on rabbit IgG-immunoglobulin light chains.

Suppression of the a2 allotype in a<sup>2</sup>a<sup>2</sup> homozygous rabbits has been obtained by the zygote transfer technique (53). In a<sup>2</sup>a<sup>2</sup> homozygous rabbits fostered in uteri of a2-immunized a<sup>1</sup>a<sup>1</sup> mothers and injected at birth with anti-a2 antisera, a2 IgG molecules synthesized by the neonatal rabbit were still not detected at 14 weeks of age. The total IgG-immunoglobulin concentration in the sera of the a2-suppressed animals was essentially the same as that found in the control a<sup>2</sup>a<sup>2</sup> animals. Thus, a compensatory increase in synthesis of a(-) heavy chains had occurred (53).

○ An allotypic specificity, designated A3l<sup>2</sup>, has recently been localized on the Fab portion of these a(-) IgG-immunoglobulin molecules (28). Oucaterlony analysis revealed that the anti-A3l antiserum reacted with some but not all rabbit sera. The sera which did react produced one, two or three precipitin bands. The A3l specificity is found in rabbits heterozygous at both the a and b loci, suggesting that these

<sup>2</sup> The lower case letter distinguishing among the loci is omitted since genetic control of the allotypic specificity designated A3l has not yet been determined.

specificities are distinct from the a or b loci. The individual specificities of the anti-A31 antiserum are currently being characterized following absorption of the antiserum with rabbit sera containing only one or two of the allotypic specificities.

The low concentration of a(-) IgG-immunoglobulins has hampered efforts to detect allotypic specificities on these molecules. However, the allotype suppression technique as described by Vice et al. (56) provides a useful method of obtaining large quantities of these molecules. Hence, it was decided to suppress animals homozygous at the a locus and to use the resulting a locus negative molecules for isoimmunization in an attempt to produce antisera which could be utilized in genetic studies.

○ In this study, the a locus of an a<sup>2</sup>a<sup>2</sup> homozygous rabbit was suppressed by the method of Vice et al. (56) in order to obtain large quantities of a locus negative IgG-immunoglobulin molecules. The animal was shown to lack any detectable a<sup>2</sup> allotypic IgG molecules for the first 30 weeks of life as detected by immunodiffusion techniques. The total IgG level in the serum of the a<sup>2</sup>-suppressed animal was essentially the same as that of a control rabbit injected with normal serum.

A new IgG-immunoglobulin allotypic determinant, designated

A50<sup>3</sup>, has been identified by isoimmunization of rabbits with these purified a locus negative IgG-immunoglobulins. The production of a precipitating antisera against the A50 allotype has permitted the classification of a large number of rabbits into two categories on the basis of presence or absence of the allotypic marker.

Immunochemical studies were utilized in the localization of the A50 determinant to the Fc portion of the heavy chain of the IgG-immunoglobulin molecule. It is our hope that these studies of a locus negative IgG-immunoglobulin molecules will provide insight into determining the complete genetic control of IgG-immunoglobulin heavy chain synthesis.

<sup>3</sup> Since allotypic specificities of rabbit immunoglobulins are given unique numbers without regard to their genetic control, the allotypic determinant identified by this antiserum was designated A50. It is not yet known if the A50 specificity is linked to the Aa, Ab or Ac locus; hence, the lower case letter, distinguishing among the loci, a, b or c, is omitted.



## METHODS AND MATERIALS

### Rabbits:

Rabbits used in these studies were obtained from several sources. Most of these rabbits were obtained from Dr. Sheldon Dray at the University of Illinois in Chicago. These rabbits are progeny from crosses of closed colonies of Flemish giants and New Zealand Whites (FG/NZW), originally obtained from the National Institute of Health. ACEP strains of phenotype a<sup>3</sup>b<sup>4</sup>c<sup>2</sup>l and C Race strains of phenotype alb<sup>4</sup>c<sup>7</sup> obtained from the National Institute of Health, and Race III rabbits of phenotype alb<sup>4</sup>c<sup>7</sup> obtained from Dr. John Albers from the University of Chicago were used for isoimmunizations. Some additional rabbits were purchased from breeders in the Chicago area.

### Suppression of a<sup>2</sup>a<sup>2</sup> homozygotes:

The a<sup>2</sup>a<sup>2</sup> donors of the zygotes were mated to a<sup>2</sup>a<sup>2</sup> bucks. At this time, the a<sup>3</sup>a<sup>3</sup> or a<sup>1</sup>a<sup>1</sup> recipients were given 2.5 mg of luteinizing hormone (Mann Research Laboratories, New York, N.Y.) intravenously to induce ovulation. After 24 to 36 hours both the donor and recipient rabbits were prepared for surgery (7), anaesthetized with Nembutal (Abbott Laboratories, Chicago, Illinois) and the ovaries exposed by flank incisions. The oviduct and uterus of the donors were exteriorized and a 30 cm length of polyethylene tubing (Intradermic, PE 160) flanged slightly at the end was inserted into the fimbriated end of the

oviduct and held in place by a miniature plastic clothespin. A blunted 20 gauge needle was inserted through the wall of the uterus and passed beyond the tubo-uterine junction into the isthmus of the oviduct. Five ml of heat deactivated rabbit serum mixed with an equal volume of saline was used for flushing the ova toward the fimbriated end of the oviduct into a watch-glass. The zygotes were examined under a dissecting scope for abnormalities and stage of division. The ovaries of the recipient were examined for ruptured follicles which must be present for a successful transplant. The donor's zygotes were drawn up in a fine glass capillary pipette and deposited 2 to 3 cm inside the fimbriated end of the recipients oviduct. A small sheet of absorbed gelatin (Gelfoam Sponge, Upjohn) was inserted before the incisions were closed off (40, 52). Once surgery was completed rabbits were placed on heating pads.

The offspring were injected intraperitoneally with a total of 10-20 ml (7-14 mg) of anti-a2 antiserum distributed at 1, 2, 3, 5, 15, 22, 31 and 40 days of age. The anti-a2 antiserum used for initial injection of rabbits was obtained from Dr. Sheldon Dray at the University of Illinois in Chicago.

Sera:

Serum was obtained from normal, immunized or suppressed rabbits at regular intervals by ear venipuncture. After the blood stood for 24 hours at 5°C, the retracted clot was removed, the remainder of the blood was centrifuged, and the serum separated. Serum was stored at -20°C until needed.

### Estimation of antigens:

Blood samples were obtained at 2 weeks of age and at regular intervals thereafter for analysis. The a1IgG, a2IgG and total IgG concentrations in the sera of the a2-suppressed a2a2 rabbit were determined for the first 18 weeks of life by radial diffusion analysis, using rabbit anti-a1, anti-a2 and goat anti-rabbit IgG Fc fragment. Purified IgG-immunoglobulins (16) were used as standards. Two ml of diluted serum in saline borate buffer were mixed with 2 ml of 3% agar in saline borate buffer at 50°C. The final concentration of the agar was thus 1.5%. The goat anti-rabbit Fc was used at a final dilution of 1 to 8, and the anti-a1 and anti-a2 at dilutions of 1 to 4. The serum agar mixture was poured into a polystyrene Petri dish 30 mm in diameter on a level table. Circular wells, 2 mm in diameter were cut 7.5 mm apart center to center. The agar from the wells was aspirated with a capillary pipette and suction. Wells were filled as uniformly as possible, level with the agar surface. Two concentrations of standard solution were included in each dish. The ring diameters were measured to the nearest 0.1 mm after 5 hours at 25°C. A standard curve was established, plotting average ring diameters (arithmetic scale) against antigen concentration in protein mg/ml (logarithmic scale) on semi-logarithmic paper. The concentration of antigens in the unknown was then read off the standard curves.

### Preparation of purified IgG-immunoglobulins:

Serum containing the a locus negative IgG-immunoglobulins used for isoimmunization was obtained from a 5 to 7 month old a<sup>2</sup>-suppressed a<sup>2</sup>a<sup>2</sup> homozygous rabbit. The a locus negative IgG-immunoglobulin molecules were purified by two sodium sulfate precipitations (18% and 14%). The protein solution was centrifuged at 10,000 RPM for 15 minutes and the supernatant removed. The precipitate was dissolved in 5 ml saline borate buffer, pH 8.1, ionic strength 0.16 and dialyzed against saline borate buffer in the cold overnight. The protein solution was dialyzed against 0.02 M sodium phosphate buffer pH 6.8 followed by recovery of the peaks from columns of diethylaminoethyl (DEAE)-cellulose in 0.02 M sodium phosphate buffer at pH 6.8 (34). The eluate was monitored on a Beckman DK2A spectrophotometer at 280 nm. Immunoelectrophoresis of the concentrated (20-40 mg/ml) IgG-immunoglobulin preparations in 1.5% Noble agar, 0.05 M barbital buffer pH 8.6, indicate that they were free of other serum proteins as detected by precipitation with anti-rabbit serum produced in goats.

### Isoimmunization of rabbits:

ACEP, Race III, C Race and FG/NZW rabbits were used for isoimmunizations. The ACEP rabbits were of phenotype a<sup>3</sup>b<sup>4</sup>c<sup>2</sup>1, while the remainder were either alb<sup>4</sup>c<sup>7</sup> or a<sup>2</sup>b<sup>4</sup>c<sup>7</sup>. The a locus negative IgG-immunoglobulins (4-8 mg) were incorporated in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) and injected intradermally and subcutaneously into

several sites of the foot pads and back of recipient, respectively. Booster doses which also contained 5 mg of protein were given at 2, 3, 4 and 5 weeks after the initial injection. Each animal received a total of 25 to 30 mg of antigen. The serum of the recipients was tested periodically with the donor serum for the appearance of precipitin bands by means of Ouchterlony analysis (34).

Analytical methods:

Double diffusion experiments were performed in 1.5% (W/V) Noble agar in 0.2 M borate, 0.15 M NaCl, pH 8.1 buffer. The Ouchterlony technique (21) was used for routine typing of the rabbit sera with appropriate reference sera for the controls. The control serum (Rabbit No. F179-3) was obtained from a rabbit whose serum coalesced with serum from the a<sub>2</sub>-suppressed animal when tested against anti-A50. Coalescence of the precipitin band of an unknown normal serum with the known reference serum was the criterion for assigning the allotype to the unknown serum.

Immuno-electrophoresis was performed on 3.25 in. by 4 in. precleaned glass slides. The glass slides were coated with 15 mm of 1.5% Noble agar in 0.05 M barbitol buffer, pH 8.6. Wells and troughs were cut and the agar was removed from the wells. The wells were then filled with the protein solution to be tested. Immuno-electrophoresis was carried out with a Buchler power supply (Fort Lee, New Jersey) at 29 ma constant current for 4 hours. Agar from the troughs were removed and

filled with appropriate antisera using a Pasteur pipette.

Goat anti-rabbit antiserum obtained from Dr. Charles Lange at Loyola University Medical Center, Maywood, Illinois was used in testing the purity of antigen preparations and in some of the controls.

Preparation and isolation of heavy and light chains:

Heavy and light chains were prepared by the method of Wilhelm and Lamm (57). Partial reduction of the IgG-immunoglobulins (260 mg) with dithiothreitol (0.06 M) was followed by alkylation with iodoacetamide (0.2 M). Following dialysis of the protein solution against 4 M guanidine-HCl buffer, the protein was filtered through a 40 x 2.8 cm. column of Sephadex G-200 in equilibrium with 4 M guanidine-HCl. The eluate was monitored on a Beckman DK2A spectrophotometer at 280 nm. Selected tubes from the heavy chain fractions were concentrated by ultrafiltration and refiltered through the G-200 Sephadex column with the same buffer. The protein eluted was immediately dialyzed against saline and concentrated by ultrafiltration. The heavy and light chain preparations were shown to be relatively free from contamination by Ouchterlony analysis against appropriate antisera (See Results). Prior to subjecting the protein to Ouchterlony analysis sodium dodecyl sulfate was added to a 0.1% concentration to the heavy chains and a 0.02% concentration was added to the agarose. The sodium dodecyl sulfate causes some non-specific precipitation lines

to form, but these could be eluted by washing the agar with saline.

Preparation of Fab and Fc fragments:

Papain digestion of purified IgG-immunoglobulins possessing the A50 determinant was performed by the method of Porter (48), as modified by Gilman et al. (20). IgG-immunoglobulin (100 mg) was incubated with 1.5 mg of mercurypapain (Sigma, St. Louis, Mo.), 0.02 M cysteine and 0.002 M disodium ethylenediamine tetracetic acid in 10 ml of 0.1 M phosphate buffer, pH 7.5, for 1 hour at 37°C. The mixture was then dialyzed in the cold overnight against 2 liters of saline-borate buffer, pH 8.1, ionic strength 0.16. The buffer was 0.03 M in borate, and 0.13 M in chloride ion (sodium crystals). Fc crystals that formed during dialysis were removed by centrifugation and washed. The supernatant protein solution was then dialyzed against cold 0.02 M sodium acetate, adjusted to pH 5.8 and diluted to 0.01 M sodium acetate concentration.

The protein was then passed through a column (2.5 X 30 cm) of carboxymethyl-cellulose (Reeve Angel, Clifton, N.J.) equilibrated with the same buffer. The eluate was monitored on a DK2A spectrophotometer (Beckman). Under these conditions Fc fragment and any undigested IgG-immunoglobulins were retained, while 70% of the Fab fragments were eluted with a volume buffer equivalent to the column buffer.

### Recrystallization of Fc Fragments:

Fc crystals were washed twice with 10 ml of 0.02 M sodium acetate and then dissolved in 0.1 M sodium acetate pH 5.0 at 37°C for 1 hour. The protein solution was dialyzed against saline-borate buffer, pH 8.0 ionic strength 0.16, in the cold room overnight. The crystals that formed were separated by centrifugation at 13,000 RPM for 15 minutes and dissolved in 0.15 M sodium acetate, pH 5.5 at 37°C for 1 hour in a water bath. The solution containing the Fc fragment did not reveal any precipitin line when tested by immunodiffusion against heavy and light chain anti-allotype antibodies directed against specificities on the Fab portion of IgG-immunoglobulin molecules, but did reveal a strong band when tested against goat anti-rabbit Fc.

### Absorption of Antiserum

Absorption of antiserum was performed by addition of 0.1 ml of serum possessing the c7 allotype, but not the A50 allotype, to 1.0 ml of unabsorbed anti-A50 antiserum. The serum mixture was incubated at 37 C for one hour, left in the cold overnight and centrifuged at 15,000 RPM for 30 minutes at 4 C. The supernatant was removed and tested for the presence of the c7 allotypic determinant by Ouchterlony analysis.



### III. RESULTS

The serum levels of a1IgG, a2IgG and total IgG were analyzed by the radial diffusion method during the first 18 weeks of development of an a<sup>2</sup>a<sup>2</sup> homozygous offspring fostered in uteri of an a<sup>1</sup>a<sup>1</sup> mother and injected neonatally with anti-a2. Ouchterlony analysis was utilized to determine the presence of a1IgG and a2IgG for the remaining 12 weeks until the animal was sacrificed at age 30 weeks. For a control, a littermate was given normal serum from an a<sup>1</sup>a<sup>1</sup> rabbit.

Rabbit A5-1 (Figure 1) was injected intraperitoneally with a total of 10-20 ml of anti-a2 antiserum (prepared in an a<sup>1</sup>a<sup>1</sup> rabbit) distributed at 1, 2, 3, 5, 15, 22, 31 and 40 days of age. The a2IgG synthesized by the neonatal rabbit was still not detected at 18 weeks of age as shown by radial diffusion analysis. In marked contrast, in the control animal (Figure 2) a2IgG was present at two weeks. It then rose rapidly in concentration and approximately 70% of the IgG-immunoglobulin molecules had the a2 allotypic specificity at all dates tested. The a1IgG present initially as a result of maternal transfer of antibodies and neonatal injection was detected until six weeks of age in both the suppressed and control animals. The total IgG-immunoglobulin concentration in the sera of the a2-suppressed animal (Figure 1) was essentially the same as that found in the control animal (Figure 2) indicating that a compensatory increase in the synthesis of a locus negative heavy chains had occurred. The serum of the a2-suppressed

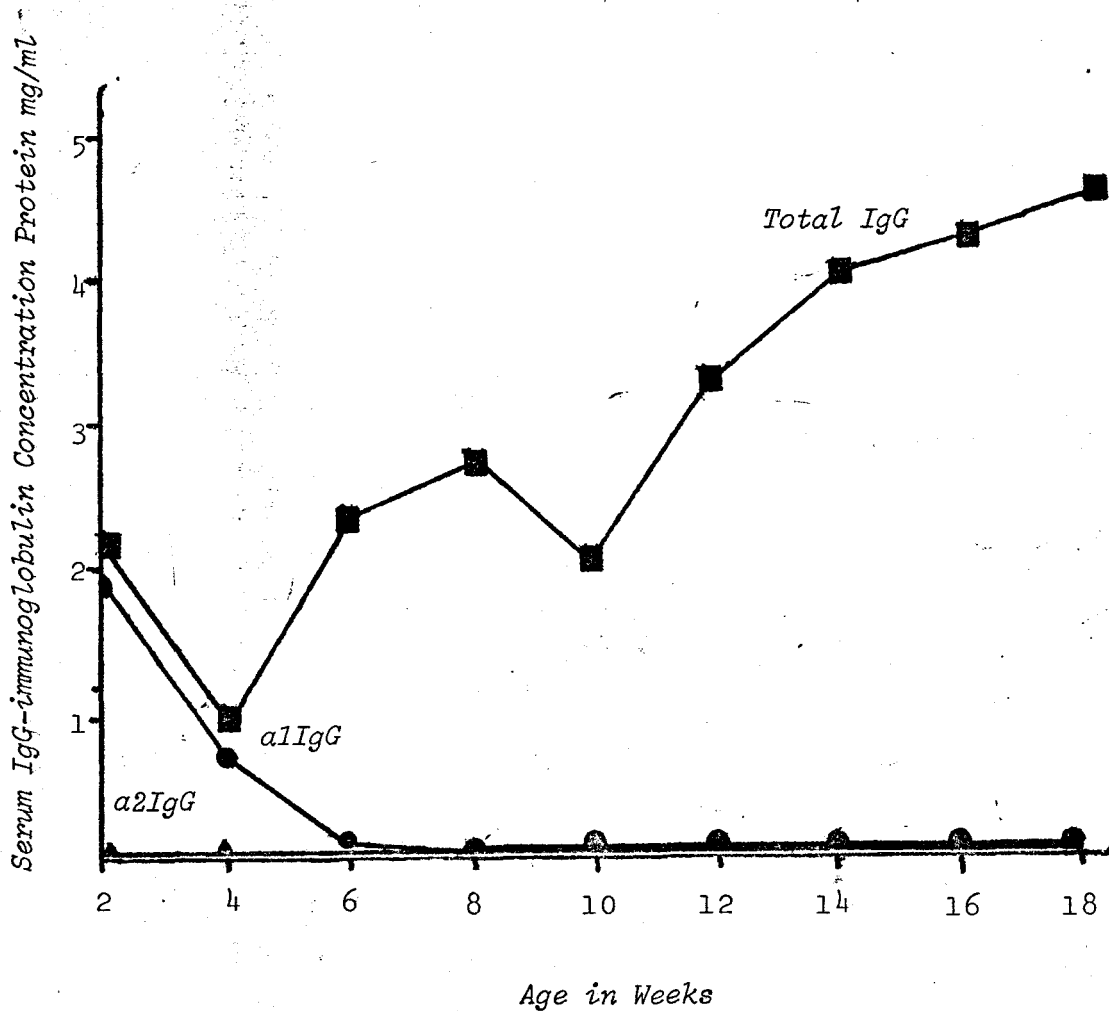


Figure 1. The a1, a2, and total IgG-immunoglobulin concentrations (protein mg/ml) in the serum of an a<sup>2</sup>a<sup>2</sup> homozygous rabbit (A5-1), after being fostered in uteri of an a<sup>1</sup>a<sup>1</sup> homozygous mother. The synthesis of a2 was suppressed by neonatal injection of anti-a2 antiserum made in an a<sup>1</sup>a<sup>1</sup> homozygous rabbit.

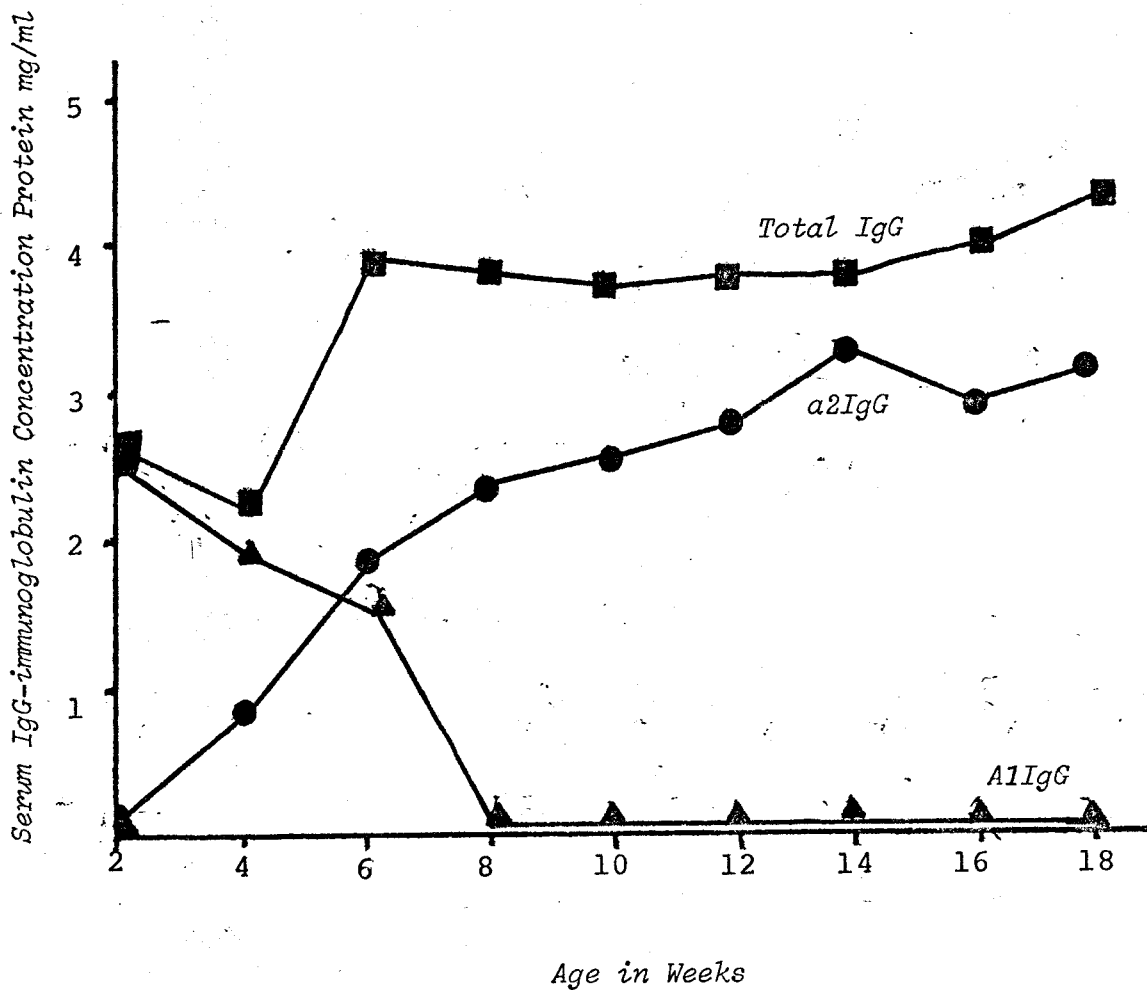


Figure 2. The  $a^1$ ,  $a^2$  and total IgG-immunoglobulin concentrations (protein mg/ml) in the serum of an  $a^2a^2$  homozygous rabbit (A5-2) after being fostered in uteri of an  $a^1a^1$  homozygous mother and injected neonatally with normal  $a^1a^1$  serum.

rabbit was subsequently tested for the presence of the a2 allotype by means of the Ouchterlony technique and no a2-immunoglobulins were detected in the suppressed rabbit until 30 weeks of age.

The IgG-immunoglobulins were isolated from the a2-suppressed rabbit by means of precipitation with ammonium sulfate followed by passage of the IgG molecules through a DEAE cellulose column. Table 1 indicates the rabbits isoimmunized with purified a locus negative IgG-immunoglobulin molecules obtained from the a2-suppressed a2b4c7 rabbit (Rabbit A5-1). The isoimmunization of rabbit 92071 (ACEP strain) of phenotype a3b4c21 resulted only in the production of antiserum directed against the c7 allotype possessed by donor serum. Rabbits D271-1 and 80361 of the NZW/FG and Race III strains, respectively, did not produce any detectable precipitating antibody.

Rabbit 55634 (C Race) of phenotype alb4c7 produced an as yet unidentified precipitating antiserum. Preliminary tests revealed that this antiserum differed from anti-a1, anti-a2, anti-a3, anti-b4, anti-b5, anti-b6, anti-b9, anti-c7, anti-c21 and anti-A50. The antiserum has not been tested against anti-A31. In immunoelectrophoresis studies, this antiserum resulted in a precipitin arc in the  $\gamma$  region when tested against serum from the a2-suppressed a2a2 homozygous rabbit (Figure 3).

TABLE 1

Production of Anti-allotype Antisera <sup>1</sup>

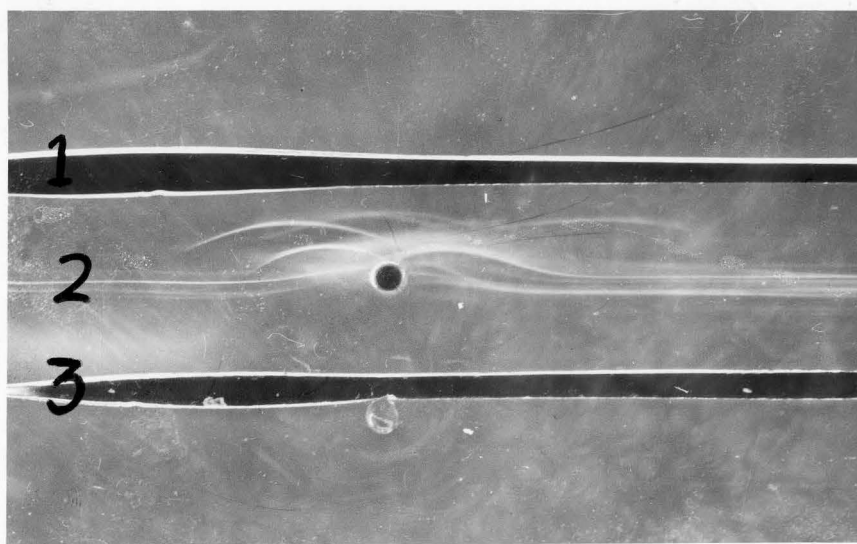
Immunized Rabbit No.	Strain	Allotypic Phenotype <sup>2</sup> of Immunized Rabbit	Antibody Specificity
92071	ACEP	a3b4c21	anti-c7
D271-1	NZW/FG	a2b4c7	No detectable precipitating antibody
80361	Race III	alb4c7	No detectable precipitating antibody
55634	C Race	alb4c7	Unidentified antiserum
70361	ACEP	a3b4c21	Anti-A50 and Anti-c7

<sup>1</sup>The recipients were injected with purified IgG from an a2 suppressed rabbit. The protein (4-8mg) was incorporated in Freund's complete adjuvant and injected intradermally and subcutaneously into several sites of the footpads and back of recipient, respectively. Booster doses containing 5mg of protein were given at 2, 3, 4 and 5 weeks after the initial injection.

<sup>2</sup>Phenotype was based on typing for a1, a2, a3, b4, b5, b6, b9, c7 and c21 only.

Figure 3. Immunelectrophoresis in 1.5% Noble agar pH 8.6 barbital buffer at constant current for 4 hours:  
1) goat anti-whole rabbit serum, 2) Aa locus-suppressed serum and 3) antiserum produced by rabbit 55634 (C Race) against Aa locus-suppressed serum.

The immunization of rabbit 15357 of the A50 strain (phenotype  $\alpha\beta\gamma\delta$ ) with purified  $\alpha$  locus negative IgG- $\gamma$  immunoglobulin resulted in the production of a precipitating antiserum which reacted to give two bands with the donor's serum and some normal rabbit sera in Ouchterlony analysis (Figure 4). Comparison of this antiserum with antiserum



Immunoelectrophoresis using the absorbed anti-A50 antiserum revealed a single precipitin arc in the  $\gamma$  region with serum from normal rabbits which possessed the A50 allotype (Figure 6). By Ouchterlony analysis, the absorbed anti-A50 antiserum gave a single diffuse precipitin line with normal rabbit sera, which coalesced with the precipitin line formed between the anti-A50 and serum from the  $\alpha$  locus negative rabbit.

In the examination of the sera of 135 rabbits, 84 (62%) were found to possess the A50 allotypic determinant (Table 2).

The isoimmunization of rabbit 70361 of the ACEP strain (phenotype a3b4c21) with purified a locus negative IgG-immunoglobulins resulted in the production of a precipitating antiserum which reacted to give two bands with the donor's serum and some normal rabbit sera in Ouchterlony analysis (Figure 4). Comparison of this antiserum with anti-a1, anti-a2, anti-a3, anti-b4, anti-b5, anti-b6, anti-b9, anti-c7, anti-c21 and anti-A31 by Ouchterlony analysis revealed that the outer precipitin line resulted from the interaction between c7 and its corresponding antibody. This was expected since the recipient lacked the c7 allotype possessed by the donor rabbit. The anti-c7 line could be specifically removed by absorption with serum from rabbits which gave only an outer (c7) precipitin line (Figure 5). The inner precipitin line resulted from the interaction between an hitherto undescribed allotype, designated A50, and its corresponding antibody.

Immunoelectrophoresis using the absorbed anti-A50 antiserum revealed a single precipitin arc in the  $\gamma$  region with serum from normal rabbits which possessed the A50 allotype (Figure 6). By Ouchterlony analysis, the absorbed anti-A50 antiserum gave a single diffuse precipitin line with normal rabbit sera, which coalesced with the precipitin line formed between the anti-A50 and serum from the a locus negative rabbit.

In the examination of the sera of 135 rabbits, 84 (62%) were found to possess the A50 allotypic determinant (Table 2).



Figure 4. Ouchterlony double diffusion analysis in 1.5% Noble Agar showing the reaction between the unabsorbed antiserum (center well) and normal alb5c7 rabbit sera (wells 1 and 2) and normal a2b4c7 serum (well 3). The wells designated C contained serum from the a2-suppressed a2b4c7 rabbit. The well designated 4 contained the serum of an A50 positive a3b4c21 animal that is producing anti-c7. The inner precipitin band is that formed by anti-A50 and its corresponding antigen, while the outer precipitin band is that formed by anti-c7 and its corresponding antigen.

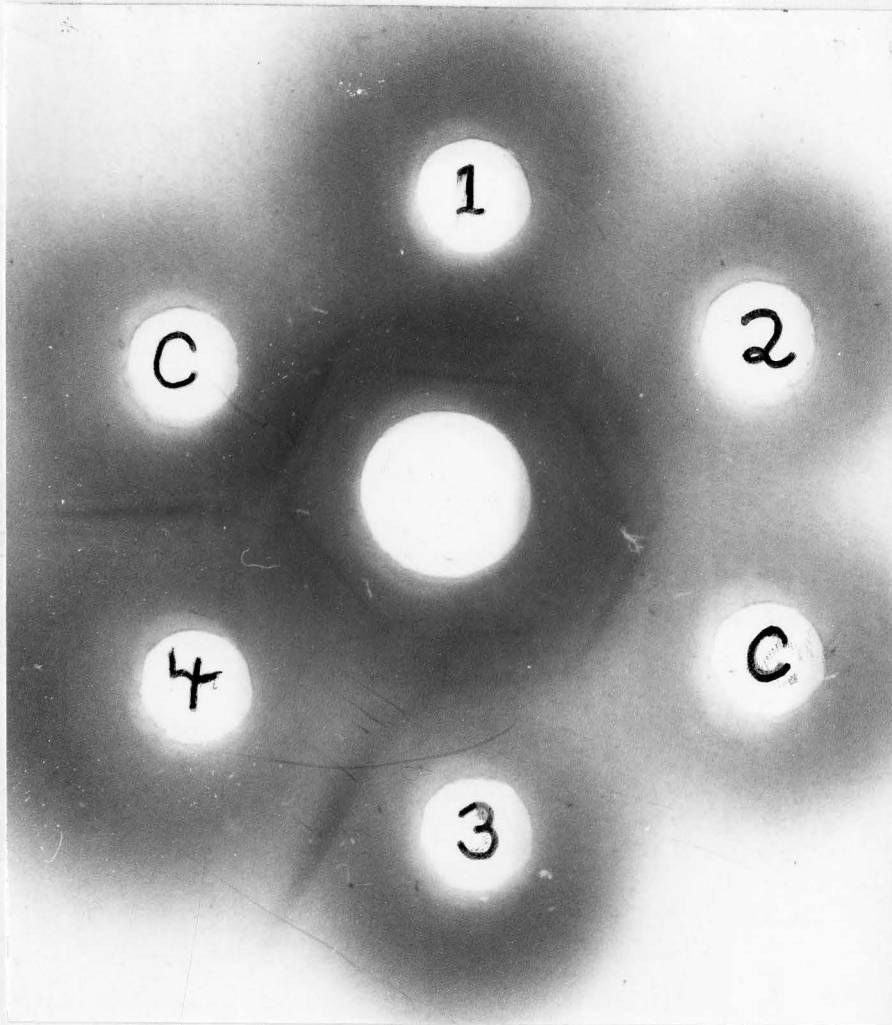
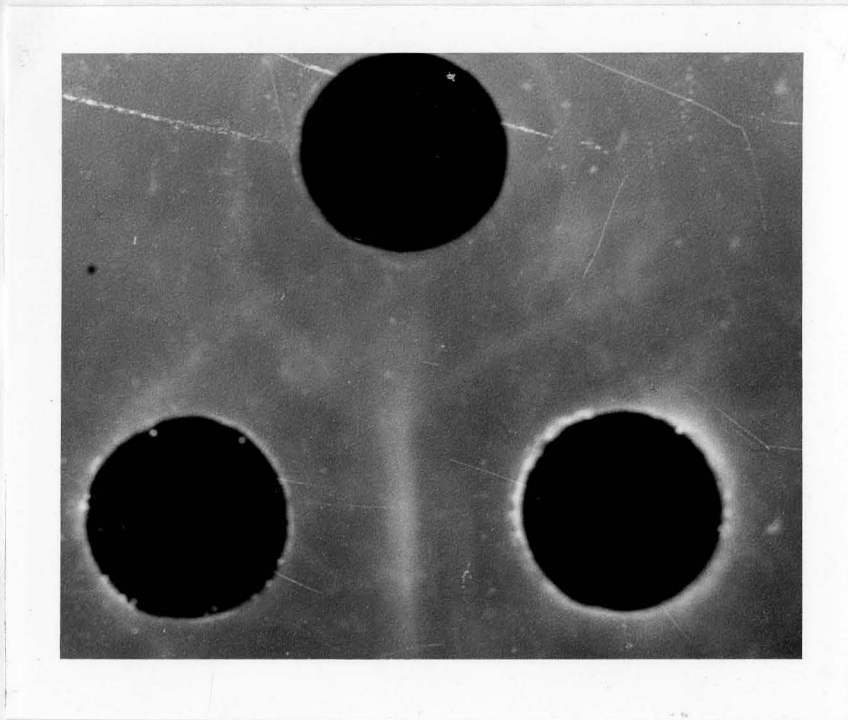


Figure 5. Double diffusion experiment in 1.5% Noble agar showing the reaction of unabsorbed anti-A50 antiserum (well 1), and absorbed anti-A50 antiserum (well 2), against serum from the a locus-suppressed rabbit (well 3).



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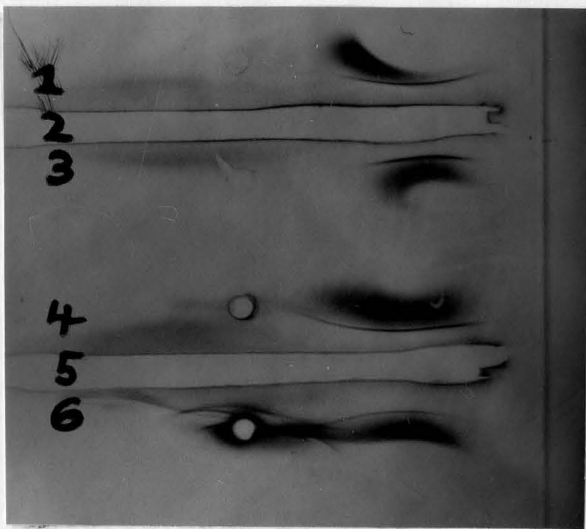
Figure 6. Immunelectrophoresis in 1.5% Noble agar pH 8.6 barbital buffer at constant current for 4 hours:

- 1) whole serum from a normal A50 positive rabbit,
- 2) absorbed monospecific anti-A50, 3) purified Aa-negative IgG, 4) purified IgG from a normal a<sup>2</sup>a<sup>2</sup> rabbit, 5) goat anti-whole rabbit antiserum and 6) normal rabbit serum. The anode is at the left.

TABLE 2

Occurrence of the A50 Determinant in Rabbits Which are Homozygous or Heterozygous for Allotypes at the a, b or c Loci

Locus and alleles	No. Tested	No. Positive	Percent Positive
a locus*			
1, 2	21	21	100
2, 3	13	13	100
b locus			
4, 5	29	21	72
4, 9	10	6	60
c locus			
7, 21	26	19	68
All Rabbits	135	84	62



\*Rabbits were included in groups as indicated, and in each case the allotypic characters of the other loci were disregarded. For example, rabbits placed in the groups as homozygous for a1 may be either homozygous or heterozygous for the b4, b5, b6, b9, c7 and c21 allotypes.

TABLE 2

Occurrence of the A50 Determinant in Rabbits Which are  
Homozygous or Heterozygous for Allotypes at the a, b  
or c Loci

Locus and Alleles	No. Tested	No. Positive	Percent Positive
Homozygous at the:			
a locus*			
<u>1</u>	35	35	100
2	32	6	17
3	23	6	26
b locus			
<u>4</u>	67	31	46
5	24	23	92
9	5	1	20
c locus			
<u>7</u>	90	59	79
21	12	5	42
Heterozygous at the:			
a locus			
<u>1</u> , 2	21	21	100
1, <u>3</u>	13	13	100
2, 3	11	3	27
b locus			
<u>4</u> , 5	29	21	72
4, 9	10	6	60
c locus			
<u>7</u> , 21	28	19	68
All Rabbits	135	84	62

\*Rabbits were included in groups as indicated, and in each case the allotypic characters of the other loci were disregarded. For example, rabbits placed in the groups as homozygous for a1 may be either homozygous or heterozygous for the b4, b5, b6, b9, c7 and c21 allotypes.

The A50 determinant appears in rabbits which are homozygous at the a, b and c loci for each allotype except the b6. Rabbits possessing the b6 allotype were not available. Note also that the A50 determinant is associated with all combinations of rabbits which are heterozygous at any of the three loci. This indicated that the locus controlling the synthesis of the A50 determinant is distinct from either the a, b or c loci.

Progeny data resulting from the three possible matings of the two phenotypes (positive or negative) indicated that the A50 determinant is heritable (Table 3). In tests of sera from progeny resulting from the matings of two parents bearing the determinant, the absence of A50 was observed in 9 of 30 animals. In tests of sera from progeny with only one A50(+) parent, the A50 determinant was present in 10 of 19 offspring. In no case was the A50 determinant detected in the progeny from two A50 negative parents. Thus, it appears that the determinant is transmitted by the usual dominant or co-dominant inheritance.

Purified IgG from an A50 positive rabbit (G203-4) was reduced with dithiothreitol and passed through a Sephadex G-200 column in 4M Guanidine-HCl in order to separate heavy and light chains. The elution pattern is shown in figure 7. The first (H) peak was shown to contain both heavy and light chains when tested against anti-Fc, anti-a1, anti-b5 and anti-c7 by Ouchterlony analysis. The second peak (L) which accounted for



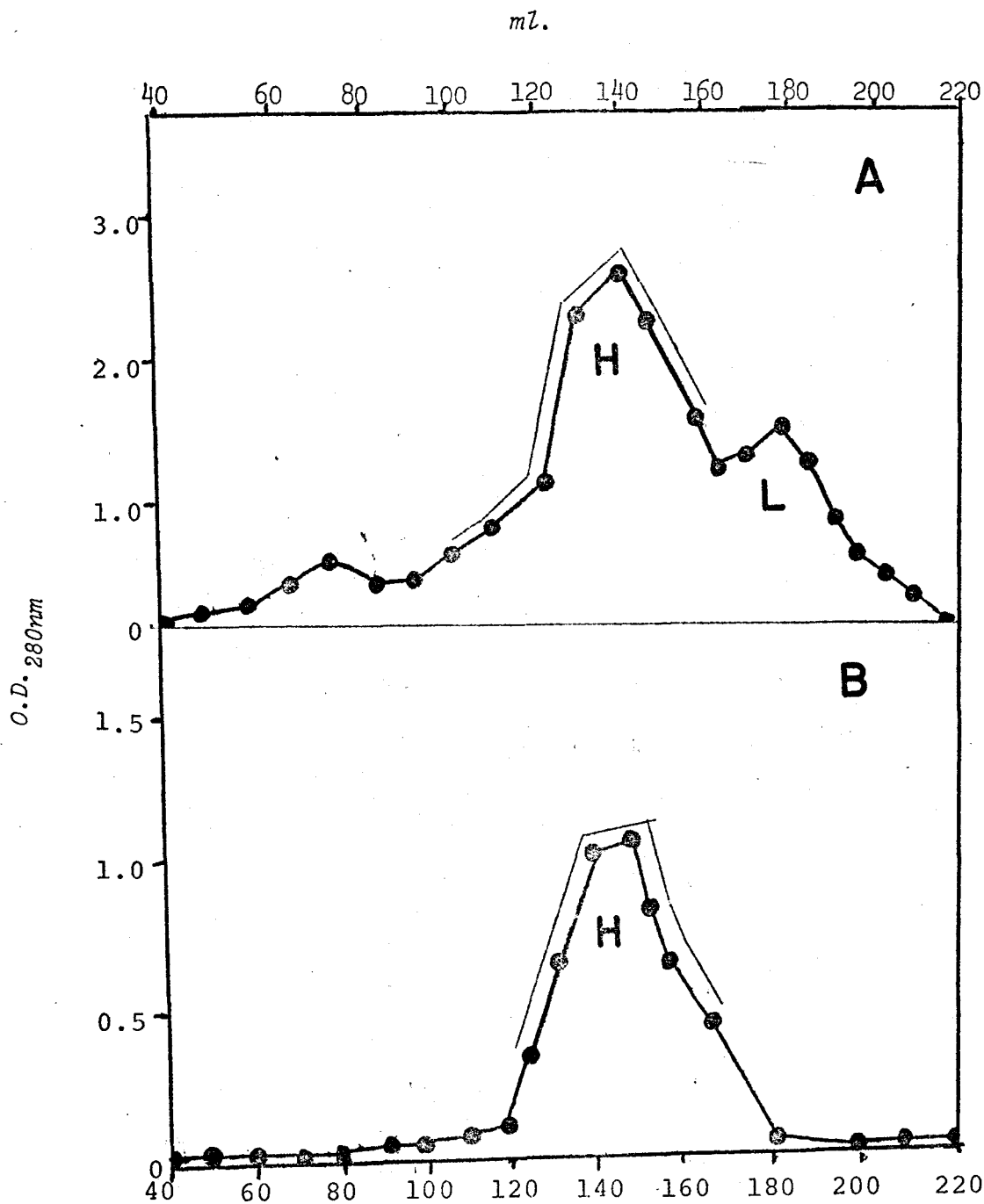
TABLE 3

Distribution of A50 Determinant in Parents and Progeny of Several Matings

Phenotype of Parents*	No. of Litters	No. of Progeny	Phenotype of Offspring	
			(+)	(-)
(+)x(+)	6	30	21	9
(+)x(-)	5	19	10	9
(-)x(-)	4	11	0	11
Total	15	60	31	29

\*There were a total of thirty parents used in these experiments, seventeen of which were positive for the A50 allotype.

Figure 7. A, Elution pattern from Sephadex G-200 in 4M guanidine-HCl buffer after reduction and alkylation of 250 mg of purified rabbit IgG-immunoglobulin. Double line indicates fractions which were combined and re-filtered. B, Second filtration of the heavy chains of panel A. Double line indicates fractions used in double diffusion studies.



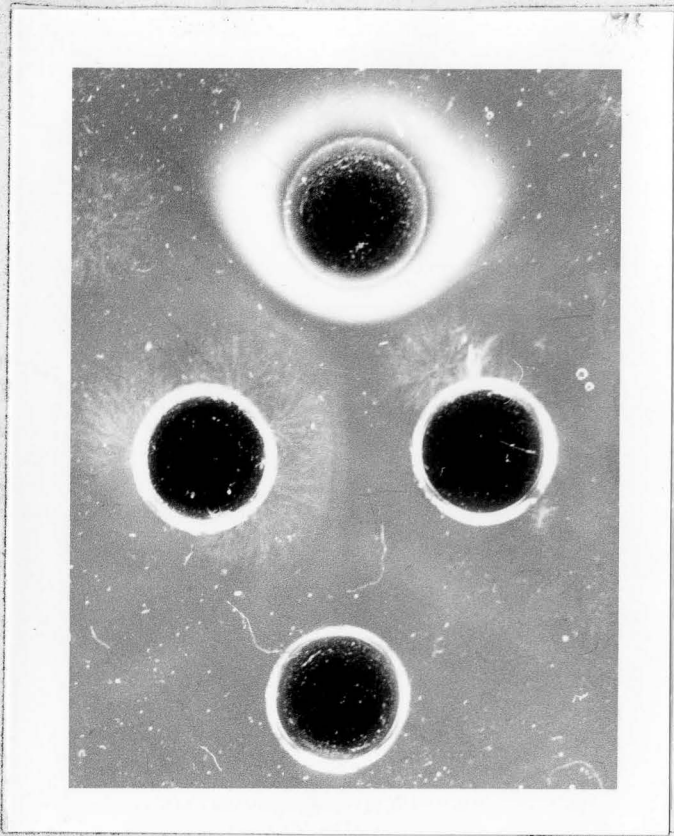
20% of the protein eluted appeared to contain no heavy chain contamination as demonstrated by Ouchterlony analysis.

Selected tubes from the heavy chain fractions were concentrated by ultrafiltration and passed again through the column (Figure 7B). Ouchterlony analysis with anti-Fc, anti-a1, anti-b5 and anti-c7 revealed that the heavy chains (7mg/ml protein) and light chain preparation (7mg/ml protein) were relatively free of contamination.

Figure 8 presents the Ouchterlony diffusion reactions of the heavy chains, light chains and control serum from the a locus suppressed rabbit against unabsorbed anti-A50. The control serum produced the typical double precipitin band while the light chain fraction formed only the outer c7 precipitin band. The heavy chain fraction formed only the inner A50 precipitin band when reacted with the unabsorbed anti-A50 antiserum. This indicates that the A50 determinant is located on the heavy chain of the IgG-immunoglobulin molecule.

Fc and Fab fragments were prepared from the purified A50 IgG molecules by digestion with papain followed by passage of the resulting fragments through a carboxymethyl cellulose column. The resulting Fab and Fc fragments (7mg/ml protein) were shown to be relatively free of contamination by Ouchterlony analysis against goat anti-IgG Fc, anti-a1, anti-b5 and anti-c7. Figure 9 presents the Ouchterlony double diffusion reactions of the Fab fragments, Fc fragments and the control a2-suppressed a2a2 serum when reacted against anti-A50 antiserum. The control

Figure 8. Double diffusion experiment in 1.0% agarose with 0.1% sodium dodecyl sulfate added showing the reaction of unabsorbed anti-A50 (with anti-c7) in well 1 against heavy chain (well H) and light chain (well L) preparations. The well designated C contained serum from the Aa locus-suppressed rabbit. The outer precipitin band resulted from the interaction of c7 and its corresponding antibody, while the inner precipitin band resulted from the interaction of A50 and its corresponding antibody.



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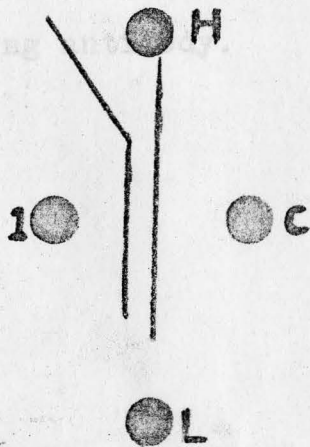
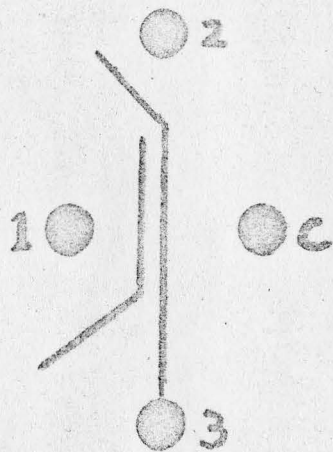
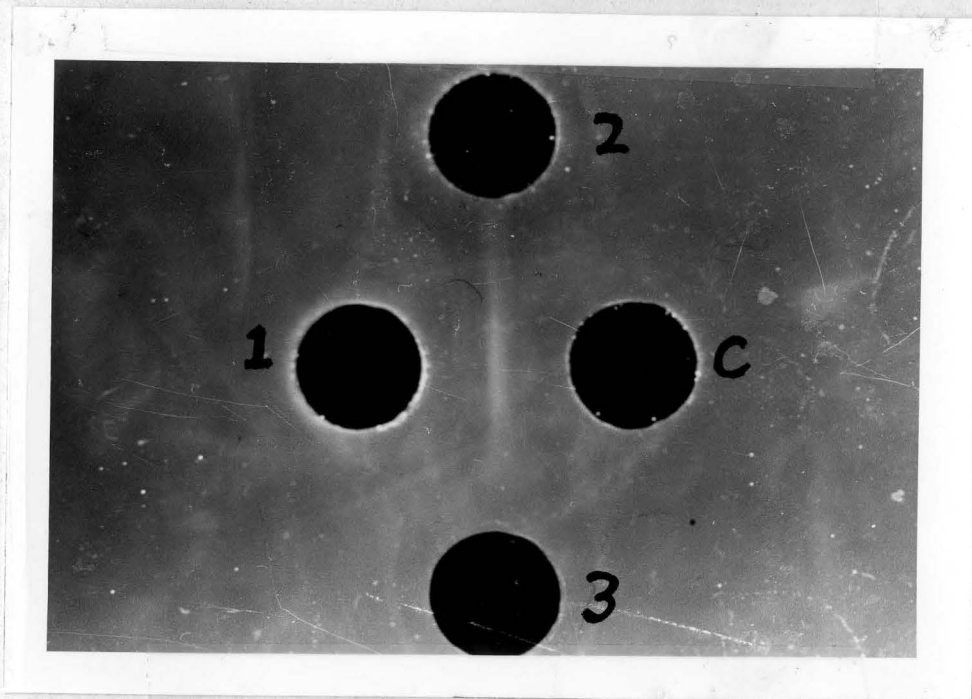


Figure 9. Double diffusion experiment in 1.5% Noble agar showing the reaction of unabsorbed anti-A50 (also containing anti-c7) in well 1 against Fab fragments (well 2) and Fc fragments (well 3). The well designated C contained serum from the Aa locus-suppressed rabbit. The outer precipitin band resulted from the interaction of c7 and its corresponding antibody, while the inner precipitin band resulted from the interaction of A50 and its corresponding antibody.





a<sub>2</sub>-suppressed serum produced the typical double precipitin band while Fab fragment formed only the outer c<sub>7</sub> precipitin band. The Fc fragment formed only the inner A50 precipitin band when reacted with the anti-A50 antiserum. This indicates that the A50 determinant is located on the Fc fragment of the IgG-immunoglobulin molecules.

## DISCUSSION

In a<sup>2</sup>a<sup>2</sup> homozygous rabbits fostered in uteri of a<sup>1</sup>a<sup>1</sup> mothers and injected neonatally with anti-a<sup>2</sup> antiserum, an inhibition of the synthesis of a<sup>2</sup>IgG and a compensatory increased synthesis of a(-) IgG immunoglobulin molecules were observed. These results are similar to those obtained by Vice et al.

(53) in homozygous a<sup>2</sup>a<sup>2</sup> rabbits fostered in uteri of a<sup>2</sup>-immunized a<sup>1</sup>a<sup>1</sup> mothers.

An allotype located on IgG-immunoglobulin molecules has been identified by isoantisera obtained from a rabbit immunized with these a locus negative IgG-immunoglobulin molecules. The allotype identified by the antiserum was designated A50.

Evidence that the A50 allotype is a determinant on IgG-immunoglobulins includes: first, the determinant was detected by antibody raised in response to isoimmunization with purified IgG-immunoglobulins; secondly, immunoelectrophoresis using the absorbed anti-A50 antiserum revealed a single precipitin arc in the  $\gamma$  region with a<sup>2</sup>-suppressed a<sup>2</sup>a<sup>2</sup> donor serum.

The A50 specificity differs from the a<sup>1</sup>, a<sup>2</sup>, a<sup>3</sup>, b<sup>4</sup>, b<sup>5</sup>, b<sup>6</sup>, b<sup>9</sup>, c<sup>7</sup>, c<sup>21</sup> and A31 (28), precipitating allotypes determined by genes at the a, b or c loci. The specificity probably differs from the A8 precipitating allotype (22, 23) since A8 is found associated with only rabbits possessing the a<sup>1</sup> allotype. The A50 specificity differs from A11 and A12 (36, 38), since these specificities are present on intact immunoglobulins but not on

separated heavy and light chains. The relationship, if any, of A50 to the A14 and A15 allotype remains to be investigated (31). The possibility exists, however, that the A50 allotypic specificity is a combination of two or more of these non-precipitating Fc allotypes.

Typing data revealed that the A50 determinant appears in rabbits which are homozygous at the a, b, and c loci, for each allotype. In addition, the A50 determinant is associated with all combinations of rabbits which are heterozygous at any of the three loci. This indicates that the A50 determinant is controlled at a locus distinct from the a, b and c loci.

Progeny data indicated that the A50 determinant is heritable. Since the determinant was not always present in progeny resulting from the matings of two parents that possess the determinant, it is probable that an additional gene is involved in the synthesis of IgG-immunoglobulin molecules. In no case has the A50 determinant been detected in the progeny from two A50 negative parents. Thus, it appears that the determinant is transmitted by the usual dominant or co-dominant inheritance.

Immunochemical studies were undertaken in an attempt to localize the A50 determinant on the IgG-immunoglobulin molecule. In studies of purified heavy and light chain preparations from A50 positive IgG-immunoglobulins, the A50 allotypic marker appeared to be associated with the heavy chain and not the light

chain. The allotypic determinant was further localized on the Fc portion of the molecule in studies utilizing papain digested IgG-immunoglobulin molecules that possess the A50 determinant.

It is presently unknown whether the A50 specificity is associated with only a locus negative immunoglobulins or is also associated with a locus positive IgG-immunoglobulins as well. Quantitative analysis of the percentage of <sup>125</sup>I labeled IgG-immunoglobulins with anti-allotype antisera would provide a means to answer this question (41).

The fact that the A50 determinant is associated with a locus negative molecules which are present in low quantities in normal rabbits may account for the inability to produce an antiserum to the A50 allotype by isoimmunization with serum from normal rabbits. In fact, the suppression of the a locus determinant and subsequent increased production of a locus negative molecules may also result in an increased production of the A50 determinant located on the Fc portion of these a locus negative molecules. Thus, it was not until the a locus specificity was suppressed and an increase in synthesis of a locus negative immunoglobulins was produced that large enough quantities of the A50 determinant could be obtained and that an antiserum could be produced by isoimmunization. This would appear to be in agreement with the results obtained with the A14 determinant (13) in which suppression of a locus animals also resulted in the suppression of the A14 determinant located on the Fc portion of

these molecules.

The immunochemical results were somewhat unexpected since the A50 determinant was localized on the Fc portion, while the suppressed a<sub>2</sub> allotype, is located on the Fd portion. The localization of a determinant on the Fd fragment of heavy chains would have been explained by assuming that the a<sup>2</sup> allele at the a locus determines a<sub>2</sub> and that another allele at a second distinct locus determines the other determinant. The hypothesis implies that a(+) and a(-) molecules represent subclasses of IgG heavy chains. This allotypic specificity may be identified following studies with the additional antiserum produced by the isoimmunization of the C Race rabbit with our a locus negative molecules. The antiserum produced by the C Race animal (Table 1) was, in fact, shown to differ from anti-a<sub>1</sub>, anti-a<sub>2</sub>, anti-a<sub>3</sub>, anti-b<sub>4</sub>, anti-b<sub>5</sub>, anti-b<sub>6</sub>, anti-b<sub>9</sub>, anti-c<sub>7</sub>, anti-c<sub>21</sub> and anti-A50 by Ouchterlony analysis. In addition, the unidentified antiserum appears to be a determinant on IgG-immunoglobulins since immunoelectrophoresis using the antiserum revealed a single precipitin arc in the  $\gamma$  region with a<sub>2</sub>-suppressed a<sup>2</sup>a<sup>2</sup> donor serum.

The finding of a precipitating allotype on the Fc fragment of IgG-immunoglobulin molecules leads us to support the recently modified theory on heavy chain synthesis (18). In this theory, synthesis of a single heavy polypeptide chain is believed to be controlled by two genes: one for the variable portion, and one

for the constant portion of the heavy chain molecule. Since the a locus specificities can be utilized as markers for the variable end, it is possible that the A50 determinant can be used as a marker on the constant portion of the heavy chain molecule in studies on the synthesis of heavy chains.

## V. ABSTRACT

The sera of an a2b4c7 rabbit (A5-1) injected at birth with anti-a2 antiserum after being fostered in uteri of an a<sup>1</sup>a<sup>1</sup> homozygous mother was analyzed for a1IgG, a2IgG and total IgG immunoglobulin concentrations by radial diffusion analysis. No IgG molecules bearing the a2 allotype could be detected until 30 weeks of age and the total IgG-immunoglobulin concentration in the serum of the a2-suppressed animal was essentially normal. Isoimmunization of a normal rabbit with the a locus negative IgG-immunoglobulin molecules obtained from the a2-suppressed a<sup>2</sup>a<sup>2</sup> homozygous rabbit resulted in the production of an antiserum that identified a new heritable allotype marker 'A50'. A large number of animals have been classified on the basis of presence or absence of the allotypic marker. The A50 allotypic specificity was shown to be located on the Fc fragment of rabbit IgG heavy chains, while absent on the light chain and Fab fragment.

### ACKNOWLEDGEMENTS

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APPROVAL SHEET

The thesis submitted by Michael I. Luster  
has been read and approved by the members of the Advisory  
Committee listed below.

The final copies have been examined by the director  
of the thesis and the signature which appears below  
verifies the fact that necessary changes have been incorp-  
orated and that the thesis is now given final approval with  
reference to content and form.

The thesis is therefore accepted in partial fulfillment  
of the requirements for the degree of Master of Science.

January 19, 1972  
DATE

Dr. John L. Vice  
Signature of Advisory Committee  
Director

Advisory Committee:

1. Dr. J. Vice (Director)
2. Dr. T. Hashimoto
3. Dr. C. F. Lange