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*GENETICS OF A GAMMA GLOBULIN ISOANTIGEN (ALLOTYPE) IN THE MOUSE**

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In man and the rabbit, isoantigens on gamma globulins have proved invaluable as structural markers in the purification and characterization of gamma globulins and their subunits.[1–3] In the mouse, in addition to these uses, the isoantigens are potentially useful as genetic markers of the whole animal and of transplanted or *in vitro* cultured lymphoid cells for genetic studies of somatic cells.[4]

In this publication, we describe studies of a mouse gamma globulin isoantigen (appearing in strains different from those already reported by Kelus[5]) and show that this isoantigen is present on antibody molecules of two different antibody specificities. We show further that the inheritance of this antigen is controlled at a single genetic locus.

Materials and Methods.—Mouse strains and sources: A/J, C57BL/6J, from Jackson Memorial Laboratories; BALB/cCrglKa, C57BL/Ka, from Department of Radiology, Stanford University; C3H/SnHz, C3H-H-2^b/SnHz, C57BL/10SnHz, from Department of Genetics, Stanford University; (101/Rl × C3H/Rl)F_1, T (noninbred tester stock), from Biology Division, Oak Ridge National Laboratory.

Isoimmunization with mouse gamma globulin: BALB/c mice, males and females, were immunized with a 50% ammonium sulfate precipitate from a pool of C57BL/6 retired breeder sera which represented more than 50 animals of mixed sex. The primary injection, 2.3 mg globulin protein per mouse, was given in Freund's adjuvant intraperitoneally and subcutaneously (1 vol of

globulin protein, 23 mg/ml, and 4 vol complete Freund's adjuvant). A recall injection of 2.3 mg globulin protein, without Freund's adjuvant, was given 3 weeks later intraperitoneally and subcutaneously. Animals were bled from the ventral tail artery at 4 weeks, and further recall injections similar to that at 3 weeks were given at monthly intervals.

Double diffusion (Ouchterlony): Both cellulose acetate strips[6] and microagar gel slides[7] were used for microprecipitin tests. The aqueous phase consisted of a Tris buffer, pH 8.9, for cellulose acetate, and phosphate buffered saline, pH 7.0,[8] for microagar slides. Microslides were allowed to develop overnight.

Hemagglutination: (a) *Sensitization of erythrocytes*—Freshly collected mouse erythrocytes were washed in saline (at room temperature) and suspended to 4% in isotonic phosphate buffered saline, pH 6.5. Equal volumes of the erythrocyte suspension and appropriately diluted mouse hyperimmune antiserum, which is reactive with the erythrocytes (e.g., C3H erythrocytes and C57BL/6 anti C3H),[8] were then incubated 30 min at room temperature followed by 30 min in the refrigerator (5°–10°C). The cells were then brought to 4°C, and while kept at that temperature, sedimented, washed, and resuspended to 2% in phosphate buffered saline, pH 6.5.

(b) *Testing of sera for anti-gamma globulin activity*—Sera to be tested were diluted $^1/_{20}$ or more in phosphate buffered saline, pH 6.5, containing 0.1% bovine serum albumin; then 0.1 ml of the diluted serum was mixed with 0.1 ml "sensitized" erythrocyte suspension in the well of a polystyrene titration plate. After incubation for 2 or more hr in the refrigerator, those wells in which agglutination occurred had an even layer of erythrocytes coating the sides and bottom of the well, whereas those wells which were negative had a round, well-defined "button" of erythrocytes at the bottom (see Fig. 1).

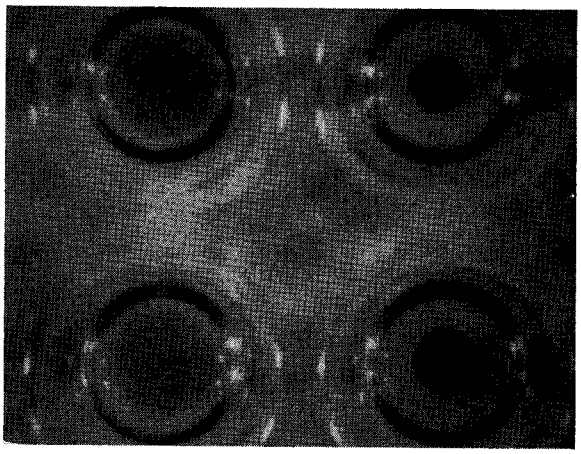

FIG. 1.—Erythrocyte Agglutination Patterns. Even coating of the bottom of the well by erythrocytes is positive. A small, round button of erythrocytes at the bottom of the well is negative.

(c) *Testing of sera for presence of gamma globulin antigen by inhibition of hemagglutination*—To test for presence of an antigen in a serum by specific inhibition of agglutination of sensitized erythrocytes with an anti-gamma globulin antiserum, 0.1 ml of an antiserum dilution at least two doubling dilutions from the titration endpoint was incubated with 1–5 μl of the test serum for 10 min prior to addition of the erythrocytes.

Genetic studies: Mice were bred 2 females to 1 male. Progeny were numbered at weaning but were not tested for gamma globulin isoantigen until 8 weeks of age.

Results.—Demonstration and location of antigen: The immunization of BALB/c mice with the "globulin" fraction of C57BL/6J serum causes production of an antiserum which reacts with C57BL/6J serum. This reaction can be demonstrated by the Ouchterlony technique (double diffusion), using either cellulose acetate or agar gel as a supporting medium. After 3 days in cellulose acetate or a few hr in agar gel, a single band of precipitation develops between the antigen and antibody wells. Further incubation does not reveal additional precipitation lines.

The pooled antiserum used for the work presented in this publication gives a visible line of precipitation when diluted as far as $^1/_{16}$ and tested against undiluted C57BL/6 normal serum, and C57BL/6 normal serum diluted $^1/_{80}$ still reacts visibly with the undiluted antiserum. For all experiments reported here, neither the antiserum nor the sera tested for antigen was diluted.

Immunoelectrophoretic analysis, in which the C57BL/6 normal serum containing the antigen(s) is allowed to migrate in the electrophoretic field and the antiserum is then placed alongside the electrophoretic pattern and allowed to diffuse, reveals a single arc of precipitation. The position of the arc (as seen in Fig. 2) indicates that

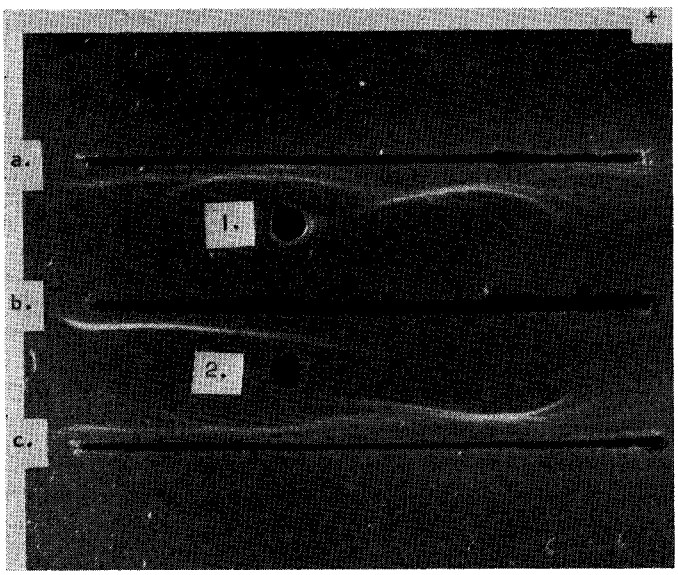

FIG. 2.—Association of Mouse Isoantigen with Gamma Globulin. Well 1, C3H (isoantigen negative) normal serum; Well 2, C57BL normal serum. Trough a and c, rabbit antimouse globulin; Trough b, BALB/c anti C57BL/Ka globulin. Supporting medium: 0.05 *M* Veronal, pH 8.2 in 1% ionagar. Electrophoresis 90 min at 6 V/cm.

the reacting C57BL/6 antigen is associated with the gamma globulins. It has therefore been designated Gg-2.[5a]

In order to demonstrate that the Gg-2 is present not only in the gamma globulins, but actually on reactive antibody molecules (usually associated with the gamma globulins), the reaction of BALB/c anti-Gg-2 with C57BL antibodies in two separate systems was studied: the absorption of anti-Gg-2 by a specific precipitate of bacteriophage with C57BL/Ka antibacteriophage and the agglutination by anti-Gg-2 of mouse erythrocytes reacted with "incomplete" isoantisera (i.e., anti-H-2 antiserum) which otherwise cause visible agglutination only in the presence of a developing agent.

(1) A specific precipitate of C57BL/Ka antibacteriophage T2 with the bacteriophage was prepared by incubating one volume of the antiserum with one volume of phage suspension (10^{13} particles/ml) for several hours at 5–10°C, washing the resultant precipitate four times, and finally suspending to one volume in phosphate buffered saline (see Fig. 3). This suspension lowers the anti-Gg-2 activity of an equal volume of the BALB anti-C57BL gamma globulin below levels detectable by immunodiffusion (at least 16-fold). Bacteriophage incubated with normal C57BL/Ka serum does not absorb anti-Gg-2 activity.

(2) The data in Table 1 show that C3H erythrocytes, reacted (and therefore coated) with C57BL/6 anti-C3H antiserum, agglutinate in the presence of BALB/c

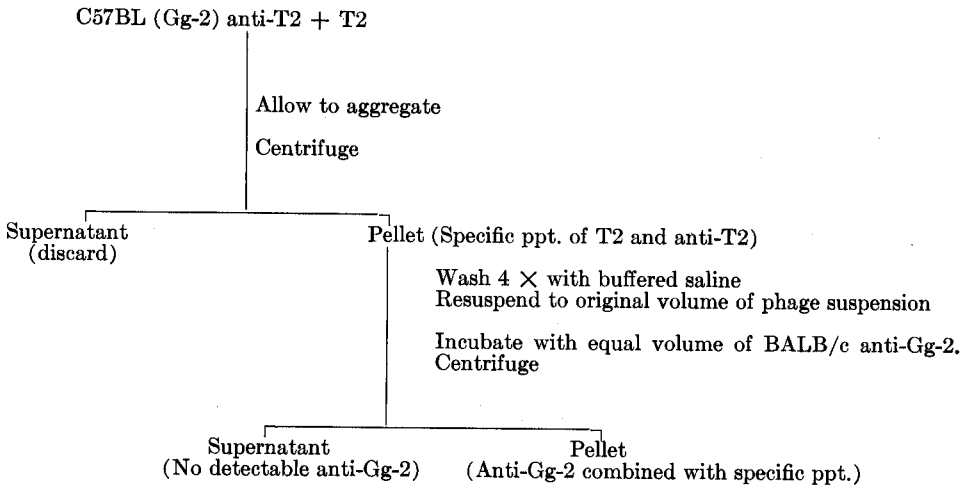

FIG. 3.—Absorption of Anti-Gg-2 with Specific Precipitate of T2 Coliphage.

anti-Gg-2 but not in the presence of BALB/c normal serum. Uncoated erythrocytes do not agglutinate in the presence of anti-Gg-2.

The hemagglutination reaction is inhibited by preincubation of anti-Gg-2 with a serum containing Gg-2 (e.g., C57BL/6J). Thus, under standard conditions (see *Methods*) 5 μl of a serum containing Gg-2, diluted as high as $^1/_{80}$, is completely inhibitory, whereas 5 μl of an undiluted serum free of Gg-2 has no effect on agglutination (see Table 2). In all cases, those sera (containing the C57BL antigen) which inhibit agglutination precipitate with anti-Gg-2 in agar and vice versa.

Two other antisera have been prepared which react with Gg-2: C3H anti-C57BL/10 and DBA/2 anti-C57BL/10. The precipitation lines formed when these sera are reacted with Gg-2 join to form bands of identity with the line made by

TABLE 1

AGGLUTINATION OF ERYTHROCYTES COATED WITH GG-2 BY BALB/C ANTI-GG-2

Serum	Erythrocytes coated with antibody carrying the Gg-2 antigen Dilution of serum used for coating	
	1/10 to 1/5120	1/10,240
BALB/c normal	−	−
BALB/c anti-Gg-2	+	−
	Uncoated erythrocytes (or exposed to nonimmune serum)	
BALB/c anti-Gg-2	−	−

Each well contains 0.1 ml of antiserum, appropriately diluted, plus 0.1 ml or of erythrocyte suspension.
+ indicates even coating of the bottom of the well by erythrocytes.
− indicates a small, round button at the bottom of the well.

TABLE 2

INHIBITION OF AGGLUTINATION OF ERYTHROCYTES COATED WITH GG-2 BY SERUM CONTAINING GG-2

Antiserum	Inhibitor-test serum	Dilution of inhibitor-test serum: Undil.	1/10	1/20	1/40	1/80	1/160	1/320
BALB/c anti-Gg-2 Diluted 1/640	C57BL/6J	$+^{i}$*	$+^{i}$	$+^{i}$	$+^{i}$	$+^{i}$	$-^{i}$	$-^{i}$
" "	BALB/cKa	$-^{i}$	$-^{i}$	$-^{i}$	$-^{i}$	$-^{i}$	$-^{i}$	$-^{i}$

*$+^{i}$ refers to presence of inhibitor; i.e., no agglutination in well.
In each well, 0.1 ml antiserum at 1/640 dilution was incubated with 5 μl of "inhibitor" serum at appropriate dilution, for ten min before 0.1 ml of Gg-2 coated erythrocytes were added.

BALB/c anti-Gg-2. Therefore, these sera were pooled and used for the genetic studies which follow.

Inheritance and distribution of Gg-2: Pooled data from a number of different outcrosses of Gg-2 positive strains, set up to determine the mode of inheritance of Gg-2, indicate that the production of the antigen is under the control of a single gene, which we designate Gg^2. In the backcross of the heterozygote to the negative parent ($Gg^2/- \times -/-$), 233 of the progeny were positive for Gg-2 and the remaining 209 were negative. This is not significantly different ($\chi^2 = 1.3$) from the ratio expected for a single factor cross (1:1, positive to negative progeny). The sex of the parent carrying the positive gene had no effect on the ratio. In the F_2 cross, where the heterozygote is intercrossed ($Gg^2/- \times Gg^2/-$), 202 progeny were positive and 78 negative. This again is not significantly different ($\chi^2 = 1.2$) from the ratio (3:1, positive to negative) expected if a single gene is segregating (see Table 3).

TABLE 3

INHERITANCE OF GG-2

Cross	Gg−2 Positive	Gg−2 Negative	Exp	χ^2	P
Backcross to negative strain (Gg−2/ − x⁻/−)	233	209	221:221	1.3	0.25
F_2 (Gg−2/ − x Gg−2/−)	202	78	210:70	1.2	0.27

Data from reciprocal matings in several strains are pooled. Negative strains are A/J, BALB/cKa, C3H/SnHz, C3H-H-2^b/SnHz, C3H/Rl, T (noninbred). Positive strains are C57BL/10SnHz, C57BL/Ka., 101/Rl.

Several mouse strains, other than the C57BL lines, react with the pooled antiserum, but the possibility that these strains produce cross-reacting antigen rather than Gg-2 has not yet been excluded. In Table 4, the results of a survey of mouse sera obtained from a number of laboratories are presented. All C57BL strains and SJL/J, LP/J, SM/J, and 101/Rl both inhibit hemagglutination and form continuous lines when placed in adjacent wells on the Ouchterlony plate.

TABLE 4

PRESENCE OF GG-2 GAMMA GLOBULIN ISOANTIGEN IN INBRED MOUSE STRAINS

Positive	Negative	
B10.D2/SnHz	A/J	MA/J
C57BL/6J	AKR/J	PL/J
C57BL/Ka	AL/N	RF/J
C57BL/10SnHz	BALB/cCrglKa	RIII/AnJ
LP/J	BDP/J	ST/J
SJL/J	BRSUNT/N	STR/N
SM/J	C3H/He	SWR/J
101/Rl	C3H/SnHz	129/Crg1Ga
	C3H.SW/SnHz	
	CBA/J	
	C57L/HeN	
	C57BR/J	
	C58/J	
	CE/J	
	DBA/1J	
	DBA/2J	
	DD/He	

Discussion.—Because of the existence of highly inbred, genetically homogeneous strains, studies on the genetic control of synthesis of gamma globulin should be more easily accomplished with mice than in man and the rabbit. Studies in these species[1−4, 9] are complicated by the fact that each individual is unique in his genetic

and antigenic constitution, whereas in an inbred mouse strain, large numbers of identical individuals are readily available as donors or recipients for immunizations or as parents for various crosses.

Kelus and Moor-Jankowski were the first to report a mouse gamma globulin isoantigen.[5] This antigen was identified in the BALB/c, C3H, and Champagne-Glaxo strains and absent in C57BL. The antigen which we describe here, Gg-2, appears in the C57BL strains but not in BALB/c or C3H. We have shown by analysis of the inheritance of Gg-2 that it is controlled by a single segregating factor. It is possible that these two mouse gamma globulin antigens represent alleles at a single locus.

The analogy with human and rabbit systems suggests that as Gg-2 gives a clear-cut line in the gamma globulin region on immunoelectrophoresis, the antigen should be present on antibody molecules. The removal of anti-Gg-2 activity with anti-bacteriophage T2 and the agglutination, by anti-Gg-2, of erythrocytes coated with Gg-2 prove this to be the case.

The immunizing antigen which we used was a 50 per cent saturated ammonium sulfate precipitate of normal C57BL serum, which contained little or no phage [$K<0.04$][10] or erythrocyte-reacting antibody; yet the anti-Gg-2 obtained was reactive against gamma globulin molecules carrying each of these antibody activities. Further, all the anti-Gg-2 was absorbed by the phage antibody precipitate, indicating that the phage antibody alone can absorb the major (and perhaps all) components of the anti-Gg-2 antiserum.

Gg-2 antigen is under the control of a single gene, as far as can be inferred from segregation ratios among several hundred mice. If two closely linked genes determined two antigenic specificities, each reactive with the anti-Gg-2 antisera, crossovers could be detected in one of two ways: (*a*) spur formation on Ouchterlony plates, or (*b*) lack of inhibition of hemagglutination by a serum which precipitates with anti-Gg-2. Sera from several hundred offspring were tested by each method, and no indication of separation of antigens was observed.

Gg-2 is found in sera of all the C57BL strains tested, as well as in sera of several other strains. These latter strains share all the antigenic determinants which differentiate C57BL from BALB/c, DBA/2, or C3H, but they may have other determinants as well.

A preliminary report of this work was given at the 31st Annual Meeting of the Genetics Society of America, August 1962. We are pleased to acknowledge the expert technical assistance of Miss Joanne Tripp and Mr. Ralph Koeth.

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[5a] Drs. Kelus and Moor-Jankowski[5] proposed the name γ^{BA} for the mouse gamma globulin isoantigen which they demonstrated. As it now appears that an extensive series of these antigens may be found, and that the same antigen may be found in different mouse strains, we prefer the use

of a more general nomenclature system, such as numbering the antigens in order of their discovery. We have named the antigen described in this publication Gg-2, to allow for the possibility that Drs. Moor-Jankowski and Kelus may wish to put their antigen in its rightful place in the series.

Note added in proof: B. Cinader and S. Dubiski in *Nature*, **197**, 705 (1963) have reported an isoantigen (designated MuA2 by them) in some of the strains carrying Gg-2. We have found a complete correlation between reaction with anti-MuA2 (kindly provided by Drs. Cinader and Dubiski) and reaction with anti-Gg-2 in all the strains we have studied. The nomenclature redundancy will undoubtedly be eliminated in the future.

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