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A SINGLE GENE CONTROLLING HEMOLYTIC COMPLEMENT AND A SERUM ANTIGEN IN THE MOUSE[1]

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A method for measuring hemolytic complement activity in mice has recently been described (1). Using this procedure, the presence or absence of hemolytic complement function has been assayed in various strains of mice. This difference in hemolytic complement activity is controlled by a single gene (2–4) which has been named Hc. The two alleles recognized to date are Hc^1 and Hc^0. Immunization of complement negative strains of mice with sera of complement positive strains has produced antisera, reacting with a β-migrating serum protein. This paper presents evidence that the presence of the serum antigen is determined by Hc.

MATERIALS AND METHODS

Mice. The following strains were initially obtained from Dr. G. D. Snell and are presently maintained either in the Department of Genetics or of Microbiology, Stanford University.

C57BL/6Jax (BL/6)
C57BL/10 SnHz
C57BL 10-$H2^d$/SnHz—old line, G12F20+ (B10.D2 old)
C57BL 10-$H2^d$/SnTa—new line, G12F3G6F2-G4F+ (B10.D2 new)
A/Jax
C3H/Sn

Progeny of the following test crosses were raised:

C57BL/10 SnHz × C57BL 10-$H2^d$/SnHz—old line (D2BF1)
D2BF1 × D2BF1 (D2BF2)
Backcross. D2BF1 × C57BL 10-$H2^d$/SnHz—old line

Normal sera. Blood was collected by tail hemisection from individual mice, the serum removed after clotting, and stored in ampules at −20°C. Sera from the inbred strains of our colonies were collected from several individuals and pooled before storage. Sera from other strains were obtained from Dr. Robert Mishell of the NIH or purchased from the Jackson Memorial Laboratories.

Antigens. a) The fraction containing the antigen was prepared by bringing pooled sera to 50% saturation with $(NH_4)_2SO_4$. The resultant precipitate was dialyzed overnight against 0.9% NaCl and reconstituted to the original volume in saline. b) Alternatively, immunization was carried out with a protein fraction of C3H/Sn serum prepared by precipitating euglobulins by dilution and subsequent purification by starch block electrophoresis carried out according to the method described by Kunkel (5).

Antisera. The antisera used were prepared by immunizing A/Jax and B10.D2 old line mice with the globulin fraction of the serum of B10.D2 new line or BL/6 mice. In addition, a partially purified C3H/Sn serum globulin fraction was used to immunize C57BL/10 SnHz mice and a rabbit. Adult mice were injected subcutaneously or intraperitoneally (i.p.) with 0.1 ml of antigen in 0.1 ml of Freund's complete adjuvant. After 2 or 3 weeks a booster injection was given. The animals were bled 5 to 7 days after the second injection. Additional injections followed by repeated bleeding were carried out at 3- to 4-week intervals. A rabbit was immunized with 1 mg o the partially purified C3H/Sn globulin fraction in 0.9% NaCl, homogenized with an equal volume of Freund's complete adjuvant, and injected into multiple intradermal sites and the footpad. A booster injection of 10 μg of the protein was given 1 week later. The rabbit was

[1] This work has been briefly reported at the Annual Meeting of the AAI, 1963.

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bled a week later and at continuing 1-week intervals.

Agar gel double diffusion and immunoelectrophoresis. The presence of the antigen in the sera of mice was detected by double diffusion with 1% agar in pH 8.2, 0.05 M Veronal buffer. Immunoelectrophoresis was performed essentially as described by Scheidegger (6). Panceau S or bromphenol blue was used for protein staining. Lipid staining was done with Sudan black (7).

Complement assays. Hemolytic complement activity was assayed on fresh sera as described previously (1).

RESULTS

The antigen related to hemolytic complement activity has been studied with three antisera. Two of these, A/Jax anti-BL/6 and B10.D2 old anti-B10.D2 new, have been prepared in mice and will be referred to as homologous antisera. The other was prepared by appropriate immunization of a rabbit and will be referred to as the heterologous antiserum. The two homologous sera were tested by double diffusion with a large number of sera of inbred strains as well as a large number of sera of individual mice of noninbred strains. The reactions of the antisera with various sera are illustrated in Figure 1. The antigens detected by these two antisera are identical since: a) they show reactions of identity on agar double diffusion and b) they have given identical results in hundreds of parallel determinations.

As shown in Tables I and II, the correlation between the presence of complement activity and the presence of the antigen was perfect in the 10 inbred strains of mice, in individual mice of two noninbred colonies, and in genetic test animals. All the members of an inbred strain of mice maintained by the proper matings are presumed to be genetically identical. It was reasonable to use pooled sera from individuals of a strain to perform the antigen and complement activity assays. The results have been confirmed by typings of many individual mice of various ages, sex and health status for both hemolytic complement and the presence of the antigen.

Of particular interest were the results obtained for the strains C57BL/10-H2^d/Sn—new line and C57BL/10-H2^d/SnHz—old line. These two strains are inbred from the offspring of two different generations in the formation of a coisogenic resistant pair (8) between C57BL/10Sn and DBA/2Jax. (The H-2^d allele of DBA/2Jax was being placed on the genetic background of C57BL/10Sn). B10.D2 old has been maintained as an inbred line after six backcross generations in the coisogenic breeding process whereas B10.D2 new comes from the 11th backcross generation. Both have the H-2^d allele of DBA/2Jax. B10.D2 old still has Hc^0 from DBA/2Jax whereas B10.D2 new has Hc^1 and the antigen from C57BL/10Sn. If the antigen and hemolytic complement activity were controlled by separate loci, they would be expected to segregate during this procedure. To test for linkage a genetic experiment was carried out. The results are shown in rows 4 and 5 of Table II. Typing o

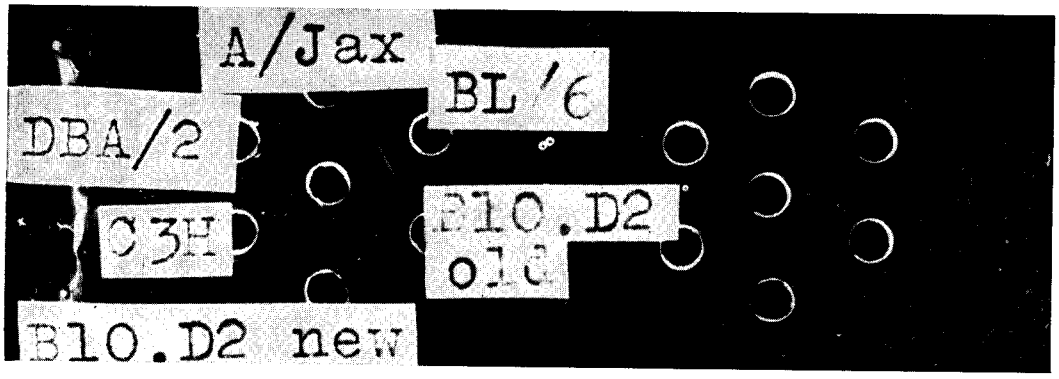

Figure 1. Macroslide double diffusion (15 μL wells separated by 3 mm) of A/Jax anti-BL/6 (left hexagon with antibody in center) and B10.D2 old anti-B10.D2 new (right hexagon) with various normal sera (set up in parallel but only labeled on left): A/Jax; BL/6, C57BL/6Jax; B10.D2 old, C57BL10-H2^d/SnHz—old line; B10.D2 new C57BL10-H2^d/Sn—new line; C3H, C3H/SnHz; and DBA/2, DBA/2Jax.

genetic test animals prepared in an attempt to find recombination between the antigen locus and the hemolytic activity locus showed complete linkage between the two (at least within 1.25 map units). The identity or very close linkage of Hc^1 and the gene controlling this antigen is supported by the data from individuals in the noninbred lines. Studies of individuals of the CF-1 and T noninbred colonies, which are segregating for Hc, showed the antigen in all sera positive for hemolytic complement activity.

Thus it has been demonstrated that all animals with hemolytic complement activity have the antigen. Mice lacking hemolytic complement might have an altered, inactive protein or be deficient in the protein. On the assumption that an altered protein would be antigenic, the serum of mice genetically deficient in complement was used to immunize complement positive mice. Although it has been easy to prepare an antiserum in B10.D2 old against B10.D2 new, attempts to make an antiserum in the positive strain against serum proteins in the negative strain have been unsuccessful. The data on these immunizations are briefly summarized in Table III.

The absence of this serum protein antigen in complement negative mice is confirmed by absorption studies with the heterologous antiserum. A monospecific serum reacting only with complement positive strains (Fig. 2) can be

TABLE I

Typings of strains of mice for hemolytic complement activity and/or the antigen

Strain	Antigen	Hemolytic Complement Activity
A/Jax	−	−
DBA/2Jax	−	−
C57BL10-H2^d/SnHz—old line	−	−
C57BL10-H2^d/SnHz—new line	+	+
B10.BY	+	+
B10.LP	+	+
C57BL/10Jax	+	+
C57BL/6Jax	+	+
C3H/SnHz	+	+
C3H.SW/SnHz	+	+
Antigen positive	C58, C57Br, DBA/1, BALB/c, 129, LP, R111, MA, BDP, SM, STR, ~~AKR,~~ 101, PL, SJL	
Antigen negative	~~CBA,~~ ST, RF, SWR, DD, BRSUNT, CE, DE	

TABLE III

Results of immunizations between mice of differing hemolytic complement activities

Type of Immunization: Serum of C′Type into C′Type[a]	No. Positive/Total after One Booster Injection	No. Positive/Total after Two Booster Injections
1) + → −	55/80	80/80
2) − → +	0/>40	0/>40
3) + → +	0/10	0/10

[a] 1) B10.D2 old injected with globulin from B10.D2 new; 2) B10.D2 new injected with globulin from B10.D2 old; 3) C57BL/10 SnHz injected with partially purified antigen from C3H/SnHz.

TABLE II

The correlation of hemolytic complement activity and the antigen in a number of mice

Type of Mice Studied	No. Positive for Antigen	No. Positive for Hemolytic Complement	No. Negative for Antigen	No. Negative for Hemolytic Complement
Inbred strains (10)	7	7	3	3
CF1 (51 individuals)	14	14	37	37
T (29 individuals)	23	23	6	6
D2BF2 (D2BF1 × D2BF1)[a] (25 individuals)	21	21	4	4
Backcross D2BF1[a] × C57BL10-H2^d/SnHz —old line (53 individuals)	32	32	21	21

[a] These crosses are described under Materials and Methods.

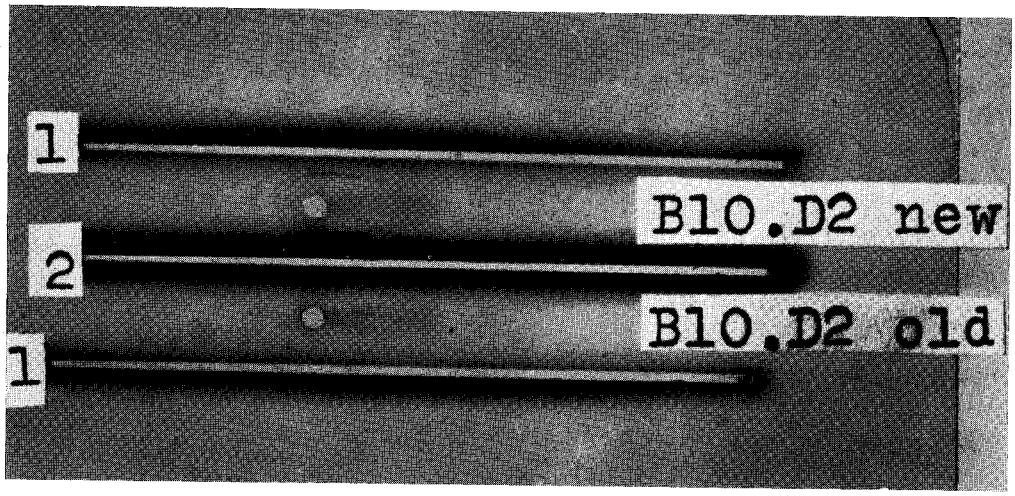

Figure 2. Agar gel of immunoelectrophoresis of B10.D2—old (C57BL10-H2^d/SnHz—old line and B10.D2 new (C57BL10-H2^d/Sn—new line) sera with B10.D2 old anti-B10.D2 new (2) in the center trough and the monospecific-absorbed rabbit anti-mouse antiserum (1) in the outer troughs for comparison. The slide was rinsed, dried, stained with Panceau S, destained and redried.

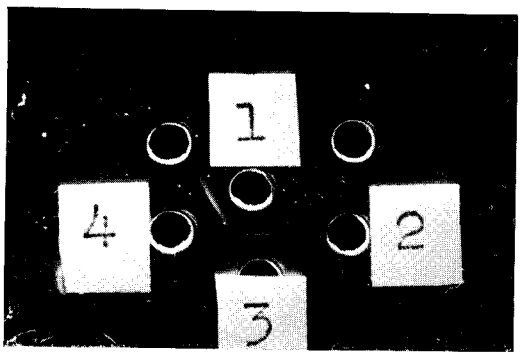

Figure 3. Agar double diffusion showing fusion of the precipitate line formed between the complement-related antigen and the isospecific and heterospecific antisera: (1) C57BL/10-H2^d/SnHz —new line; (2) B10.D2 old anti-B10.D2 new; (3) heterospecific antiserum absorbed with DBA/2Jax; and (4) A/Jax anti-BL/6. The precipitation line between (3) and (4) consists of the minor component of the A/Jax anti-BL/6 reacting with the DBA/2Jax serum used to absorb the heterospecific antiserum.

prepared by absorbing the heterologous antiserum with complement negative sera, e.g., B10.D2 old. The monospecific component cannot be absorbed by incubation with an excess of complement negative serum and gives a line of fusion with B10.D2 old anti-B10.D2 new as shown in Figure 3. We conclude that the rabbit antiserum detects the complement-related antigen. It does not detect a related protein in complement negative mice.

Ten C57BL/10 SnHz mice immunized with the partially purified C3H/Sn antigen showed no antibody after two booster injections. Thus, the C3H/Sn hemolytic complement antigen is unlikely to have antigenic determinants different from the homologous protein in C57BL/10 SnHz.

In order to determine the electrophoretic characteristics of the antigen, immunoelectrophoresis was carried out. Figure 2 shows the immunoelectrophoretic pattern of A/Jax anti-BL/6 with a complement positive serum, B10.D2 new line (B10.D2 old line serum is in the adjoining well). The pattern indicates that the antigen is a β-migrating serum protein. The line was not stained by Sudan black, suggesting that it is lipid negative.

The A/Jax anti-BL/6 antiserum is not monospecific. Two minor antigen components of mouse serum react with it. They may be seen as very weak lines when the appropriate mouse serum is reacted with this antiserum in a sensitive double diffusion test.

DISCUSSION

There has been much interest in the purification and characterization of components of comple-

ment (9). The availability of various strains of mice all with a deficiency in hemolytic complement activity determined by one genetic locus, permitted immunochemical studies of the deficient component. Antisera prepared in mice negative for hemolytic complement activity against serum proteins of mice positive for complement detects an antigenic protein whose presence is determined by the locus Hc or a locus very closely linked to it. Further evidence for the identity of a protein determined by the Hc locus and this antigen is: a) the antibody inhibits hemolytic complement (4) and, b) the β-migrating fraction from starch block electrophoresis of complement positive serum, which is in itself devoid of hemolytic complement activity, supplements the serum of genetically negative mice (10).

Generally, serum antigens detected by isoimmunization have been found to be part of a polymorphic system. That is, the animals immunized are not deficient in the serum protein but have an antigenic variant of it (11). Although the animals are immunologically tolerant to most of the injected protein, they are capable of reacting to that part of the protein which contains antigenic determinants different from their own. It seems unlikely that complement negative mice have a corresponding β-globulin which lacks hemolytic complement activity. The ease with which a complement negative strain forms antibodies against serum of a complement positive strain known only to be genetically different at this one locus (Hc), whereas the inverse immunization is completely unsuccessful, suggests that the protein is missing in complement negative animals. The absorbed heterospecific antiserum would be expected to detect more and different antigenic sites than the homologous antisera. Yet the heterospecific antiserum does not detect any cross-reacting protein in the sera of mice negative for hemolytic complement activity. Antigenic polymorphism among complement positive mice for this serum protein might yet be detected by additional attempts at isospecific immunization between complement positive animals.

An antigen presumably involved in complement activity has previously been detected in mouse sera by heterologous antisera (12–14). This is not the antigen here discussed as the heteroantigen is present in strains negative for hemolytic complement activity (e.g., DBA/2Jax).

SUMMARY

Mice of strains deficient in hemolytic complement have been immunized with sera of strains positive for hemolytic complement activity. The resulting antisera react only with sera from mice with hemolytic complement activity. Typing of a variety of individuals for both the serum antigen and complement activity indicate that the same locus, Hc, determines the two. An *absorbed* rabbit antiserum prepared against the partially purified antigen also reacts only with serum from mice positive for hemolytic complement activity. This suggests that the allele at the complement locus, previously described as Hc^0, determines no recognizable product.

REFERENCES

1. Rosenberg, L. T. and Tachibana, D. K., J. Immun., **89:** 861, 1963.
2. Herzenberg, L. A., Tachibana, D. K., Herzenberg, L. A. and Rosenberg, L. T., Genetics, **48:** 711, 1963.
3. Tachibana, D. K., Ulrich, M. and Rosenberg, L. T., J. Immun., **91:** 230, 1963.
4. Tachibana, D. K. and Rosenberg, L. T., Folio Microbiol., In press.
5. Kunkel, H. G. and Trautman, R., *Electrophoresis*, Edited by M. Bier, p. 225, Academic Press, New York, 1959.
6. Scheidigger, J. J., Int. Arch. Allergy, **7:** 103, 1955.
7. Uriel, J. and Grabar, P., Ann. Inst. Pasteur, **90:** 427, 1956.
8. Snell, G. D., J. Nat. Cancer Inst., **21:** 843, 1958.
9. Mayer, M. M., *Experimental Immunochemistry*, Edited by E. A. Kabat and M. M. Mayer, p. 204, Charles C Thomas, Springfield, 1961.
10. Unpublished data.
11. Steinberg, R. G., Progr. Med. Genet., **2:** 1, 1962.
12. Clausen, J. and Heremans, J., J. Immun., **84:** 128, 1960.
13. Müller-Eberhard, H. J. and Nilsson, U., J. Exp. Med., **111:** 217, 1960.
14. Williams, C. A. and Wemyss, C. T., J. Exp. Med., **114:** 311, 1961.