DISTRIBUTION, INHERITANCE, AND PROPERTIES OF AN ANTIGEN, MUB1, AND ITS RELATION TO HEMOLYTIC COMPLEMENT

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Individuals of the same species may differ from one another in protein composition, these differences being of two types: in the first type most of the peptide structure of a particular protein is identical in all individuals except for a limited region of the molecule where differences in certain amino acids may occur. This is exemplified by the polymorphism of hemoglobin, pseudocholinesterase, haptoglobin, and of gamma globulin (1). The individual differences in the latter class of proteins, the gamma globulins, have been revealed by isologous antibody and are designated as allotypes (2, 3).

Individuals of the same species may differ from one another also by a second type of variation, consisting in the presence or absence of certain proteins. Where this is the case, it may be expected to have detrimental consequences as, for example, in various clotting deficiencies, such as congenital afibrinogenemia, hemophilia, Christmas Disease, absence of factors 5, 7, 8, and 10 (4, 5), and by such metabolic deficiencies as total albinism (tyrosinase), Von Gierke's disease (glucose-6-phosphatase), familial goitrous cretinism (iodotyrosine deshalogenase), phenylketonuria (phenylalanine hydroxylase), and Cugler-Najjar syndrome (glucuronyl-transferase) (6, 7). It should be stressed however, that the deficiencies mentioned may be either due to total failure to synthesize the molecule or to loss of function, resulting from alteration of the molecule.

This paper is concerned with antigen MuB1 (8), a member of the second type of variation in protein composition. The existence of this variation was revealed initially by an antibody produced by immunizing certain inbred strains of mice with the serum of other inbred strains of mice (8, 9). Using this MuB1 antibody, we have surveyed, by agar diffusion, sera from more than a hundred mouse strains to determine the distribution of MuB1 in inbred mice and also its inheritance in cross-bred mice. In addition, we have examined the serum from about 85 species belonging to 15 different orders of mammals and found that MuB1 is widely distributed in quite unrelated families. Finally, data will be presented suggesting that MuB1 is connected with complement, is linked with

hemolytic complement (Hc) (10), and may be one of the components of serum complement.

Materials and Methods

Agar Gels.—All gels were made with purified agar, certified (0560-01) (Difco Laboratories, Inc., Detroit).

Coating of surfaces: Glass surfaces were coated with 0.5 per cent (w/v) agar, made up in glass-distilled water.

Single diffusion: 6 gm of agar were dissolved in 1 litre of glass-distilled water containing 4.3837 gm NaCl, 2.04135 gm KH_2PO_4 8.5182 gm Na_2HPO_4, and 1 gm of sodium azide (pH 7.5).

Double diffusion: Double diffusion was carried out in one of the two following preparations: (*a*) 1.5 gm of agar per 100 ml were made up with glycine buffer adjusted to pH 8.4, I = 0.05 (3.5649 gm glycine, 2.7787 gm NaCl, 50 ml 0.1 N NaOH in 1 litre). (*b*) 0.7 gm agar, 8.5 gm NaCl, 25 gm glycine, and 8.25 gm sodium diethylbarbiturate were made up to 1 litre with glass-distilled water. The pH was adjusted to 7.4–7.6 (private communication, Dr. C. L. Christian).

Immunoelectrophoresis: Agar was diluted in veronal buffer (0.05 M sodium diethylbarbiturate, 0.01 M diethylbarbituric acid, and 0.05 M sodium acetate) pH 8.6, ionic strength 0.1).

Immune Sera.—Antiserum to human complement component C'1 (11) was obtained from Dr. I. H. Lepow and Dr. J. Pensky, of Western Reserve University, Cleveland.

Rabbit antiserum to human β_1-C was obtained from Dr. K. Lange, New York University Medical College, New York.

Rabbit antiserum to the complement factor in human S11 globulin was obtained from Dr. C. L. Christian, Columbia University, New York (12).

Rabbit antiserum to α_2-macroglobulin of the rat was obtained from Dr. P. Grabar, Villejuif, Seine, France.

Dr. B. Blumberg, National Institutes of Health, Bethesda, gave us human Ag(a+) antibody (13).

Antiserum to mouse myeloma protein was obtained from Dr. J. L. Fahey, National Institutes of Health, Bethesda.

Antisera to various other human serum proteins were purchased from Hyland Laboratories, Los Angeles.

Mouse Sera.—Generally, mice were obtained from the breeders and then bled in our laboratory. However, sera of the following strains of mice were furnished by the investigators listed in Table I: Af/MySp, BRVR/Sr, BSVS/Sr, DBA_t/A, DBA/HeA, DBA/LiA, DBA/S, GFF, IF/Bcr, JU, MaS/A, PHH, PHL, PS, WH/Ht.

Lyophilized samples of sera were obtained of the following strains (see Table I): DDK, KK, and NC.

Bleeding of Mice.—Mice were bled from the tail. Each animal was introduced into a plastic centrifuge tube with an air hole punched through the bottom and was held in place by a neoprene stopper which had a central hole, through which the mouse's tail was threaded. The tail was then immersed in warm water, dried with filter paper, and the end snipped off. Blood was collected and allowed to clot and separate at room temperature. 3 to 6 hours later the blood was centrifuged at +2°C and the serum was frozen. For experiments involving complement assays, blood was shed into a 2 ml test tube immersed in an ice-water bath. The tube and bath were placed into a conical flask which was evacuated. The tubes containing blood were kept in an ice-water bath for about 1 hour, and were then centrifuged at 0–2°C. The serum

was then separated and was centrifuged again until free of erythrocytes. Sera were used immediately after separation.

Immunization of Mice.—Mice were immunized by subcutaneous injection with antigen incorporated in complete Freund's adjuvant.

Immunization of Rabbits.—Rabbits were injected subcutaneously with precipitates prepared from mixtures of MuB1-positive serum and antiserum to MuB1 of mouse origin. These precipitates were incorporated in Freund's adjuvant.

Absorption of Antisera (Rabbit).—Various quantities of MuB1-negative mouse sera or pseudoglobulin fractions obtained from MuB1-positive mouse sera were added to the antisera obtained from rabbits. The mixtures were kept at 37°C for 1 hour and precipitates were removed by centrifugation at +2°C.

Preparation of Euglobulin.—(*a*) Sera were dialysed at +2°C against glass-distilled water for 2 to 3 days. The precipitate which formed was resuspended, the dialysis bags were emptied into centrifuge tubes, and the precipitate was collected by centrifugation. The supernatant was brought to the desired volume by pervaporation at +4°C or by dialysis against polyethylene glycol and then dialysed against 0.15 M NaCl. (*b*) Alternatively, glass-distilled water, cooled to +2°C, was saturated with CO_2 and 10 volumes were added to 1 volume of serum,, and the mixture was centrifuged at +2°C. Precipitates and supernatants were treated as described above.

Double Diffusion in Agar.—Glass slides 18.5 x 10 cm, were covered with melted solutions of 0.5 per cent agar which was allowed to set. The slides were then placed in an air oven at 37°C, until the gel had dried as a thin transparent film (14). Onto the slides was poured 10 ml of molten agar (0.7 or 1.5 per cent). The agar was allowed to set at room temperature. Cups were cut in the gel with a punch. The punch was made of stainless steel tubings (diameter 2.5 mm) attached to a brass disk; holes in the brass disk allowed displacement of air from the tubes. The punch consisted of a central tube and 6 peripheral tubes, arranged in a hexagon. The distance between the axis of the central tube and the axis of the peripheral tubes was 5.75 mm. The plates, covered with agar gel, were then placed on wetted filter paper which covered the bottom of plastic boxes. After the cups had been filled with the appropriate reactants the boxes were closed with tightly fitted lids. When precipitin zones were observed with an immune serum the specificity of the reaction was checked by a parallel test in which normal sera of the same strain as the immune sera were put into corresponding cups.

The quantity of antigen was estimated by a dilution method in which sera containing the antigen were diluted in a geometrical progression. The central cup was filled with an antiserum to MuB1 and the dilutions of antigen were placed into the peripheral cups. The highest dilution giving a discernible zone, was taken as the end point.

Single Diffusion in Agar (14).—In single diffusion experiments glass tubes were used which were 76 mm in length, 1.5 to 1.6 mm internal diameter, and 2.8 to 3.1 mm outer diameter. These tubes were cut from Natelson micro-blood collecting tubes obtained from Clay-Adams Inc., New York. Serum was mixed with an equal volume of melted 0.6 per cent agar solution. This mixture was kept at 56°C. Tubes were filled with these solutions up to the bottom $\frac{2}{3}$ of the tubes. The bottom of the tubes was then closed with plasticine. The agar was allowed to set; the antigen solution was introduced into the $\frac{1}{3}$ top of the tube. The tops of the tubes were then closed with plasticine. The tubes were placed vertically in a tightly closed box, kept at 22.2°C. The tubes were incubated for 7 days, then the agar columns were photographed (15). The distance between interphase and antigen-antibody zone was measured. The distance so obtained was compared with the distance obtained when various dilutions of a standard serum (DBA/1J, male, 6 months old) were allowed to diffuse into agar-antibody mixtures.

Immunoelectrophoresis was carried out with equipment obtained from LKB-Produkter AB,

Stockholm, Sweden. The methods used were those given in their operating manual (1-6800A-EO2, immunoelectrophoresis equipment).

After single, double diffusion, and immunoelectrophoretic separation, zones in agar were photographed under darkground illumination (15).

Treatment of Serum with Hydrazine.—A solution of 0.15 M hydrazine was adjusted with HCl to pH 7.5 and 0.2 ml was added to 1.0 ml of euglobulin solution. The mixture was then incubated at 37°C and samples were withdrawn at intervals. Immediately after these withdrawals, 0.1 ml of propionaldehyde solution (0.15 M, pH 7.5) was added to 0.3 ml of the mixture of serum and hydrazine.

Treatment of Euglobulin Solutions with Ammonia.—1.4 ml of euglobulin solution was mixed with 0.35 ml of aqueous solution containing 0.15 M NH_4OH. The mixtures were incubated at 37°C and samples of 0.2 ml were withdrawn at intervals. To these samples, 0.04 ml of 0.15 M HCl was added.

Assay of Complement. Volumes of serum equal to 0.1, 0.05, 0.02, and 0.01 ml were delivered into pyrex tubes (10 x 75 mm) held in an ice bath and made up to 0.1 ml by addition of veronal buffer (Kabat and Mayer, 1961, reference 16). To each mixture was then added 0.05 ml of a suspension of sheep erythrocytes (1.25 per cent v/v) which had been sensitized immediately beforehand with a very large amount of antibody (hemolysin) as suggested by Rosenberg and Tachibana, 1962, reference 17). When used for the assay of guinea pig complement, 1.25 per cent sensitized sheep cells were prepared by mixing equal volumes of 2.5 per cent washed sheep erythrocytes and 1/8000 hemolytic serum. However, for mouse complement, it was necessary to mix a 1/10 dilution of the hemolytic serum with 2.5 per cent cells in order to make the final concentration of sensitized cells 1.25 per cent (v/v). After adding the sensitized cells to the diluted mouse sera, the mixtures were incubated with periodic shaking for 1 hour at 37°C, whereupon 1.0 ml of veronal buffer was added to each mixture and the tubes were centrifuged for 10 minutes at 1500 RPM and at 1°C. The degree of hemolysis was determined from the concentration of unhemolysed cells, because of the complication of hemoglobin contributed by the mouse sera which frequently were hemolysed. To estimate unhemolysed cells, the supernatants were poured off and the residues of unhemolysed cells were deliberately hemolysed by adding 3.0 ml distilled water. The optical densities of the tube contents were then measured at $\lambda = 410$ mμ. Control mixtures, in which 0.1 ml veronal buffer had been used in place of the dilution of mouse serum, were included in each experimental series; red cells were centrifuged, lysed, and optical densities were measured. The optical density of these control mixtures was taken as representing 0 per cent hemolysis during the incubation phase of the test. If the residual cells from test mixtures gave optical densities of close to this control value, the mouse sera were considered to lack detectable complement. Dilutions of mouse sera which resulted in an optical density of hemolysed residual cells, equal to one-half of the control values were considered to contain one 50 per cent hemolytic unit (HU_{50}).

Fractionation of Hemolytic Antiserum.—Hemolytic antiserum, which had been prepared by the short-term immunization procedure of Darter (18) was separated into 3 fractions by column chromatography on sephadex G-200 using the method of Roskes and Thompson (19). The 3 fractions contained, in order of elution, proteins of molecular weight greater than 200,000, proteins of molecular weight in the region of 150,000, and proteins of molecular weight around 70,000 or less. As found by tests with guinea pig complement, the major part of the original hemolytic antibody activity occurred in the first (macroglobulin) peak, with a much smaller amount in the second peak, and no activity in the fluid. The three fractions, which were highly diluted relative to the starting serum, were concentrated by dialysis against carbowax until their volume was only ten times that of the starting serum.

RESULTS

Isologous Antibody and Distribution of the Antigen MuB1.—We have previously reported the presence of an antigen, MuB1, in some inbred strains of mice (8, 9). This antigen was identified by means of an isologous antibody. The antibody was obtained by immunizing inbred mice of strains DBA/2J and A/HeJ with serum obtained from mice of strains DBA/1J and C57L/J, respectively. Immune sera elicited in mice of the two different strains gave identical positive and negative reactions in double diffusion with the sera of various inbred strains of mice. A survey was made of the distribution of MuB1 amongst sera from some 70 inbred strains. The antigen was found to be fairly widely distributed and to be present in some 61 per cent of the inbred strains of mice tested (Table I). The remarkable fact that of two otherwise very similar strains, such as DBA/2 and DBA/1, one lacked and the other possessed the antigen MuB1, led us to examine various strains derived from DBA. It was our aim to investigate whether any strain derived from DBA/1 had lost the antigen MuB1, whether any strains derived from DBA/2 had acquired the antigen, and whether strains derived from DBA, before its division into DBA/1 and DBA/2, possessed or lacked the antigen MuB1.

It will be seen from Table II that all strains derived from DBA/1, including coisogenic strains, possessed the antigen MuB1, with the exception of strain DBA/1$_o$Hu. All strains derived from DBA/2 lacked the antigen. Strains designated DB or DBA and presumably derived from the original DBA strain, prior to its division into DBA/1 and DBA/2, either possessed (58 per cent), or lacked (42 per cent), the antigen. The percentage of MuB1-positive strains was remarkably similar to that of all the inbred strains of animals.

In addition to the inbred mice examined, all of 40 Swiss mice from the Connaught Laboratories and from our own colony were found to possess the antigen under investigation. Amongst Swiss mice from Manor Farms, Staatsburg, New York, a small percentage of animals lacking MuB1 was found. In general, it seemed that non-inbred Swiss mice tended to possess the antigen MuB1.

The question next arose whether there was a qualitative or quantitative difference between MuB1 in different MuB1-positive strains of mice. We tested this by determining the amount of MuB1, by single diffusion in 6- to 7-week-old individuals of five inbred strains of mice, and found a remarkable homogeneity in the concentration of MuB1 (Table III).

Status of MuB1-Negative Mice.—The question next considered was whether MuB1-negative animals possessed a macromolecule which corresponded to the antigen detected in MuB1-positive mice. We asked ourselves whether the antibody to MuB1 was directed to a few sequences of amino acids which were present in the protein of some individuals but were absent in the corresponding protein of other individuals, or

TABLE I

The Distribution of the Antigen MuB1 among Inbred Strains of Mice

Strain	No. positive / No. tested	Source	
		Investigator	Institution
A/J	0/8*	PES‡	Roscoe B. Jackson Memorial Laboratories, Bar Harbor
A/HeJ	0/7*	"	" "
AKR/J	0/8*	"	" "
AU	0/12*	Willys K. Silvers	The Wistar Institute, Philadelphia
BALB/cJ	8/8*	PES	Roscoe B. Jackson Memorial Laboratories
BDP/J	2/2*	"	" "
BRVR/Sr	8/8*	Howard A. Schneider	The Rockefeller Institute
BSVS/Sr	8/8*	" " "	" " "
BUA/Wi	0/8*	J. Walter Wilson	Brown University, Providence, Rhode Island
BUB/Bn	4/4*	Seldon Bernstein	Roscoe B. Jackson Memorial Laboratories
BUB/Bn-*C*	4/4*	" "	" "
BUB/Wi	8/8*	J. Walter Wilson	Brown University
BUC/Wi	0/7*	" " "	" "
BUE/Wi	0/8*	" " "	" "
CBA/J	8/8*	PES	Roscoe B. Jackson Memorial Laboratories
CE/J	0/7*	"	" "
C3Hf/BiOci	2/2*	A. A. Axelrad	Ontario Cancer Institute, Toronto
C3H/HeJ	7/7*	PES	Roscoe B. Jackson Memorial Laboratories
C3H/HeN	4/4	Harold H. Hoffman	National Institutes of Health, Genetics Unit, Laboratory Aids Branch, Bethesda
CHI/St	8/8*	L. C. Strong	Roswell Park Memorial Institute, Biological Station, Springville, New York
C57BL/HaOci	1/1	A. A. Axelrad	Ontario Cancer Institute
C57BL/6J	6/6§	PES	Roscoe B. Jackson Memorial Laboratories
C57BL/10J	8/8§	"	" "
C57BR/cdJ	9/9*	"	" "
C57L/J	8/8*	"	" "
C58/J	9/9*	"	" "
DBA/1J	6/6*	"	" "
DBA/2J	0/6*	"	" "
DDK	0/10*	Takeshi Tomita	Nagoya University School of Agriculture, Nagoya-Shi, Japan
DM/Ms	0/4*	Kazuo Moriwaki	National Institute of Genetics, Yata 1, III, Misima, Sizuoka-Ken, Japan
F/St	6/6*	L. C. Strong	Roswell Park Memorial Institute, Biological Station
FAKI	0/7*	J. F. A. P. Miller	Pollards Wood Research Station, Chester Beatty Research Institute, Buckinghamshire, England
FU	8/8*	Liane B. Russell	Oak Ridge National Laboratory, Oak Ridge, Tennessee
GFF	0/12*	P. W. Muggleton	Glaxo Research Ltd., Greenford, Middlesex, England
HR/De	4/4§	Margaret Deringer	National Institutes of Health, National Cancer Institute
IF/Bcr	0/7*	June Marchant	University of Birmingham, Birmingham, England
I/FnLn	0/6*	John B. Lyon, Jr.	Emory University, Atlanta, Georgia
JU/Fa	0/8*	D. S. Falconer	Institute of Animal Genetics, Edinburgh, Scotland
KK	0/10*	Takeshi Tomita	Nagoya University School of Agriculture
MA/J	2/2*	PES	Roscoe B. Jackson Memorial Laboratories
MaS/A	0/8*	O. Müllbock	Antoni van Leeuwenhoek-huis, Amsterdam, Holland
MO/Ko	9/9*	N. Kobozieff	Ecole Nationale Veterinaire d'Alfort, Laboratoire de Genetique, Alfort, France
NBL/N	0/6§	Harold A. Hoffman	National Institutes of Health, General Biology Section

* MuA2-negative (21, 22).

‡ Pedigreed Expansion Stocks.

§ MuA2-positive (21, 22).

TABLE I—*Concluded*

Strain	No. positive / No. tested	Source	
		Investigator	Institution
NC	0/10*	Takeshi Tomita	Nagoya University School of Agriculture
NS/Fr	0/5§	F. Clarke Fraser	McGill University, Department of Genetics, Montreal, Canada
NZB/Bl	0/7*	W. K. Lane Petter	Laboratory Animals Center, M. R. C. Laboratories, Carshalton, Surrey, England
NZO/Bl	8/8*	J. C. Kile, Jr.	Cumberland View Farms, Clinton, Tennessee
PE/R1	9/9*	Liane B. Russell	Oak Ridge National Laboratory
PHH	0/6§	J. A. Weir	University of Kansas, Department of Zoology, Lawrence, Kansas
PHL	8/8*	" " "	" "
P/J	3/3*	PES	Roscoe B. Jackson Memorial Laboratories
PL/J	4/4*	"	" "
PS	9/9*	J. Mouriquand	Centre d'Etudes Nucleaires de Grenoble, Grenoble, France
RF/J	0/8*	PES	Roscoe B. Jackson Memorial Laboratories
RIII/AnJ	4/4	"	" "
RIII/J	4/4*	"	" "
RIII/WyJ	4/4	"	" "
SEA/Gn-se	8/8*	Earl L. Green	" "
SEC/1Gn	8/8*	Margaret C. Green	" "
SJL/J	6/6§	PES	" "
SL/R1	8/8*	Liane B. Russell	Oak Ridge National Laboratory
SMA/Ms	0/3*	Kazuo Moriwaki	National Institute of Genetics
SM/J	3/3§	PES	Roscoe B. Jackson Memorial Laboratories
ST/J	0/3*	"	" "
STOLI/Lw	7/7*	Lloyd W. Law	National Institutes of Health, Carcinogenesis Section
SWR/J	0/8*	PES	Roscoe B. Jackson Memorial Laboratories
T6	7/7*	K. G. Millican	Pollard's Wood Research Station, St. Giles, England
WH/Ht	10/10§	H. Hewitt	Mount Vernon Hospital, Northwood, Middlesex, England
YBR/HeWiHa	0/10*	Theodore S. Hauschka	Roswell Park Memorial Institute, Buffalo, New York
129/J	5/5*	PES	Roscoe B. Jackson Memorial Laboratories
2BC3H	3/3*	Commercial stock	Simonsen Laboratories, Inc., Gilroy, California
2C3H	3/3*	" "	" "

whether the antibody was adapted to many determinants of a molecule which was present in some and completely absent in other animals. This type of problem is very difficult to resolve and has posed itself quite frequently in blood group studies, where the presence of an antigen has often been revealed many years after the corresponding antigen in other individuals has been detected.

We attempted to raise antibody against a hypothetical antigen of MuB1-negative animals. To this end, we immunized MuB1-positive animals, C57L/J and Connaught Swiss mice, with the serum of RF/J and DBA/2J mice, respectively. Though these immunizations were continued for 9 to 12 months, we did not succeed in eliciting an antibody. This failure is attributable either to the absence of such a corresponding antigen, or could also be connected with the properties of the animals chosen for immunization.

Since attempts to raise an antibody to an antigen corresponding to MuB1 had failed and therefore had led to inconclusive results, we attempted to examine the question of the antigenic status of MuB1-negative mice by means of antibody to MuB1 raised in an animal of another species and of another order. Such an antiserum may be expected to contain antibodies not only to determinants by which individual mouse

TABLE II

The Distribution of the Antigen MuB1 among Inbred Strains of Mice Derived from DBA Stock

Strain	No. positive / No. tested	Source	
		Investigator	Institution
DBA/1J	6/6	PES	Roscoe B. Jackson Memorial Laboratories
DBA/1fBHu	8/8	Katherine Hummel	" "
DBA/1-H-2^b (D1LP)	8/8	George D. Snell	" "
DBA/1-H-2^e(D1.C)	8/8	" " "	" "
DBA/1_oHu	0/12	Katherine Hummel	" "
DBA/1Hu	10/10	" "	" "
DBA/1JSn	7/7	George D. Snell	" "
DBA/2J	0/10	PES	" "
DBA/2	0/14	Margaret Deringer	National Institutes of Health
DBA/2eBDe	0/11	" "	" " " "
DBA/2DeHu	0/5	Katherine Hummel	Roscoe B. Jackson Memorial Laboratories
DBA/2DeJ	0/10	PES	" "
DBA/2JN	0/12	Harold A. Hoffman	National Cancer Institute, Bethesda
DBA/2Sp	0/10	William L. Simpson	Detroit Institute of Cancer Research, Detroit
DBA/2WyDi	0/19	Margaret Dickie	Roscoe B. Jackson Memorial Laboratories
DBA/Ch	0/7	H. B. Chase	Brown University
DBA/Ep	8/8	Carlos E. Epper	Commision Nacional de Energia Atomica, Buenos Aires, Argentina
DBA/Ha	10/10	Theodore S. Hauschka	Roswell Park Memorial Institute
DBA/HaOci	7/7	Arthur A. Axelrad	Ontario Cancer Institute
DBA/HeA	0/4	O. Mühlbock	Antoni van Leeuwenhoek-huis
DBA/K1	8/8	K. E. Hellström	Karolinska Institutet, Stockholm, Sweden
DBA/LiA	7/7	O. Mühlbock	Antoni van Leeuwenhoek-huis
DBA_f/A	4/4	" "	" " " "
DBA/R1	0/8	Liane B. Russell	Oak Ridge National Laboratory
DBA/S	0/8	Kurt Stern	University of Illinois, Department of Pathology, Chicago
DBA/Sp	8/8	William L. Simpson	Detroit Institute of Cancer Research
DBA_f/Sp-D	8/8	" " "	" "
DBA/Sp-p	7/7	" " "	" "
DB/Sp	0/6*	" " "	" "

proteins differ but also to the determinants which occur in the antigen MuB1 and in a corresponding hypothetical antigen in MuB1-negative mice. One would therefore assume that such an antibody to MuB1 would show some reactivity with an antigen similar to MuB1, if present in MuB1-negative mice (20). To explore this, we immunized rabbits with a carefully washed immune precipitate separated from a mixture of mouse serum containing MuB1 and mouse antiserum to MuB1 (9). The resulting immune serum could be shown by immunoelectrophoresis to react with several serum proteins.

The pseudoglobulin fraction of MuB1-positive mice does not contain demonstrable quantities of molecules of MuB1 specificity. When such pseudoglobulin fractions were added to rabbit antisera, all but one of the antibodies, in this serum, were removed. The remaining antibody, directed against a single antigen, was tested against the serum of 20 different strains of inbred mice and reacted with those and only with those sera which had been shown, with antibody of mouse origin, to contain the antigen MuB1. The absorbed rabbit serum and the

TABLE III

The Relative Concentration of MuB1 in the Serum of Male Mice of Five Inbred Strains

Strain	Age	Concentration*
	wks.	
BALB/cJ	7	0.465
		0.465
CBA/J	6	0.465
		0.465
C3H/HeJ	6	0.465
DBA/1J	7	0.339
		0.476
		0.465
		0.488
		0.541
		0.444
SJL/J	6	0.488
		0.465

* Concentration measured by single diffusion and expressed as a fraction of the concentration of MuB1 in the serum of male DBA/1J mice, 6 months of age.

mouse MuB1 antisera reacted in double diffusion with the same antigen (Fig. 1). By single diffusion, it could be shown that the quantity of rabbit antibody to MuB1 was not reduced by the absorption with the murine pseudoglobulin fraction of MuB1-positive mouse serum.

If the serum of MuB1-negative animals contained an antigen which had in part the same peptide structure as MuB1, rabbits would be expected to make antibody to the determinants common to MuB1 and to a hypothetical corresponding macromolecule in MuB1-negative animals. It should, therefore, be possible to remove a portion of the rabbit antibody to MuB1 by addition of the serum of MuB1-negative animals. The multispecific rabbit antiserum was therefore absorbed with the serum of DBA/2J mice. The absorbed antiserum con-

tained only antibody to MuB1. It was shown by single diffusion that the quantity of antibody to MuB1 was not reduced by absorption with serum from mice of strain DBA/2J. Thus, whether the heterologous rabbit antiserum was absorbed with MuB1-positive sera from which the antigen MuB1 was removed, or whether it was absorbed with MuB1-negative serum, there was no appreciable change in the quantity of antibody reacting with MuB1.

The foregoing findings are compatible with the view that MuB1-negative mice do not synthesize an antigen which has appreciable structural similarity with the antigen MuB1.

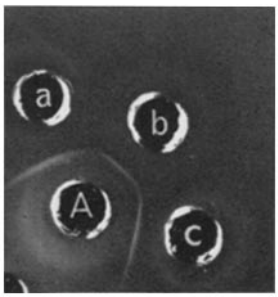

FIG. 1. Double diffusion test showing reactions between three MuB1 antisera and a MuB1-positive normal mouse serum. *A*, normal serum from mouse of strain DBA/1J; *a*, antiserum to MuB1 from mice of strain A/HeJ; *b*, antiserum to MuB1 from mice of strain DBA/2J; *c*, antiserum to MuB1 from rabbit. (This serum was absorbed with a pseudoglobulin fraction of MuB1-positive mouse serum.)

We have, so far, examined the status of MuB1-negative mice in terms of the specificity of a rabbit antibody to the mouse antigen MuB1 and have found that this antibody does not react with antigen in the serum of MuB1-negative mice. We next turned to a more indirect approach to this question, based on the comparison of the specificity of isologous and heterologous antibody to MuB1. We first examined the specificity of the murine antibody. If MuB1-negative animals synthesized an antigen corresponding to MuB1, one would expect the antibody to MuB1 to show a very narrow range of specificity. This is indeed generally found to be the case with allotypic antibodies. We have observed it in the case of murine antibodies to the mouse allotype MuA2 (21–23). This antibody did not react with gamma globulin of any mammal tested, not even with the gamma globulins of such closely related rodents as the rat.

For the tests of the cross-reactivity of the antibody to MuB1 we employed antibody raised in mice of strain A/HeJ which was much more potent than the antibody raised in mice of strain DBA/2J. We found that the antibody to MuB1 interacted with the sera of mammals of most of the orders tested (9), except *Marsupialia* and *Tubulidentata*. The serum of several species of fish, amphibia, reptiles, and birds did not react with the antibody to MuB1.

An antigen reacting with the murine antibody to MuB1 was found in the serum of species belonging to 13 of the 15 orders of mammals tested and amongst 63 of a total of 85 species whose serum was examined. The antibody reacted equally with the sera of all normal rabbits and also with sera of rabbits deficient in complement (24). It reacted with the serum of all humans tested, including the serum of thirty Eskimos and twenty Amerindians from the Queen Charlotte Island Indian Reserve. There were a few species, such as minks, in which the serum of some individuals gave a reaction with the antibody and that of others did not. There were also a few mammalian species, such as moose and deer, which did not have an antigen which reacted with the mouse antibody, but in general, the majority of mammals possessed the antigen which reacted with the antibody to the mouse antigen (Table IV). Thus, the antibody to MuB1 reacted with corresponding antigen of other mammals in a very different way to that observed with allotypic antibodies.

The extent of cross-reactivity does depend on the quantity, as well as the quality of the antibody. The quantity of antibody to MuB1, raised in DBA/2 mice by immunization with serum from DBA/1 mice, was only 16 per cent (double diffusion dilution test) of that elicited in strain A/HeJ. This antiserum showed correspondingly less extensive cross-reactivity, but nevertheless, zones with sera of a few distantly related species of animals, such as lynx, goat, and dog, were observed.

The generally observed, narrow specificity of allotypic antibody may be attributed to the structural similarity between the antigen used for immunization and an antigen present in the animal being immunized. If this assumption is correct, the cross-reactivity of antibody to MuB1 may be attributed to the absence of an antigen sharing structural features with MuB1, in MuB1-negative animals. In order to test this assumption further, we examined the specificity of antibody to MuB1 raised in the rabbit, an animal belonging to an order (*Lagomorpha*) other than *Rodentia*. The quantity of the antibody, so obtained, was 40 per cent of that elicited in mice in strain A/HeJ and 200 per cent of that elicited in mice of strain DBA/2J. It showed less reactivity with rodents than either of the murine antibodies and none with mammals of other orders. It gave cross-reaction with rat and hamster sera, only. Thus, antibody to the mouse protein MuB1, raised in an animal of another order (*Lagomorpha*) than the mouse, had a narrower specificity range than antibody raised in the mouse (order *Rodentia*).

On the basis of the foregoing experiments, it is reasonable to conclude that MuB1-negative animals lack the entire molecule on which the MuB1 specificity resides. These animals are consequently not tolerant to any portions of the MuB1 molecule including those polypeptides which, having not undergone evolutionary changes, are found on the isofunctional protein of other mammals. As a consequence, the mouse antibody can react with the corresponding serum

TABLE IV

The Distribution in Mammals of Antigens Cross-Reacting with Murine Antibody to MuB1

Order	Family	Genus	Species	No. positive / No. tested	Common name
Marsupialia	Macropodidae	*Macropus*	*rufus*	0/2	Kangaroo
Insectivora	Erinaceidae	*Erinaceus*	*europaeus*	2/2	Hedgehog
Chiroptera	Phyllostomidae	*Sturnira*	*lilium*	0/1	Yellow-shouldered bat
		Uroderma	*bilobatum*	0/1	Yellow-eared bat
		Artibeus	*lituratus*	1/2	Fruit bat
		Artibeus	*jamacensis*	2/2	Fruit bat
		Artibeus	*cinereus*	0/1	Fruit bat
	Vespertilionidae	*Myotis*	*lucifugus*	1/13	Little brown bat
		Eptesicus	*fuscus*	0/3	Big brown bat
	Molossidae	*Molossus*	*ater*	0/1	Bonneted (mastiff) bat
Primates	Lorisidae	*Galago*	*senegalensis*	1/1	Bush baby
	Cebidae	*Alouatta*	*villosa*	0/1	Howler monkey
	Cercopithecidae	*Macaca*	*mulatta*	12/12	Rhesus monkey
	Hominidae	*Homo*	*sapiens*	80/80	Man
Edentata	Bradypodidae	*Choloepus*	*hoffmanni*	1/1	Two-toed sloth
Pholidota	Manidae	*Manis*		1/1	Pangolin
Lagomorpha	Leporidae	*Lepus*	*europaeus*	1/1	European hare
		Sylvilagus	*floridanus*	10/10	Cottontail
		Oryctolagus	*cuniculus*	69/69	Domestic rabbit
Rodentia	Sciuridae	*Tamiasciurus*	*hudsonicus*	2/2	Red squirrel
		Marmota	*monax*	16/16	Woodchuck
	Castoridae	*Castor*	*canadensis*	1/1	Beaver
	Cricedidae	*Oryzomys*		0/1	Rice rat
		Peromyscus	*maniculatus gracilis*	12/12	Woodland deer mouse
		Sigmodon	*hispidus*	10/10	Cotton rat
		Cricetulus	*barabensis*	4/4	Chinese hamster
		Mesocricetus	*auratus*	4/4	Syrian hamster
		Ondatra	*zibethicus*	0/2	Muskrat
		Microtus	*pennsylvanicus*	1/1	Meadow mouse
	Muridae	*Rattus*	*norvegicus*	4/4	Norway rat (white)
	Erethizontidae	*Erithizon*	*dorsatum*	4/4	Porcupine
	Caviidae	*Cavia*	*porcellus*	77/81	Guinea pig
Cetacea	Physeteridae	*Physeter*	*catadon*	1/1	Sperm whale
	Delphinidae	*Tursiops*	*truncatus*	0/1	Bottlenose porpoise
Carnivora					
Suborder: Creodonta	Canidae	*Canis*	*familiaris*	10/10	Domestic dog
	Procyonidae	*Procyon*	*lotor*	26/26	Raccoon
		Nasua	*narica*	0/1	Coatimundi
	Mustelidae	*Mustela*	*vison*	28/30	Mink
		Mustela	*furo*	3/3	Ferret
		Martes	*americana*	1/1	Marten
		Mephitis	*mephitis*	13/13	Skunk
	Felidae	*Felis (Lynx)*	*canadensis*	3/3	Canada lynx
Suborder: Pinnipedia	Phocidae	*Callorhinus*	*ursinus*	4/4	Fur seal
		Phoca	*groenlandica*	1/1	Harp seal
		Phoca	*vitulina*	1/1	Harbour seal
Tubulidentata	Orycteropodidae	*Orycteropus*	*afer*	0/1	Aardvark
Proboscidea	Elephantidae	*Loxodonta*	*africana*	1/1	African elephant
		Elephas	*elephas*	0/1	Indian elephant
Sirenia	Trichechidae	*Trichechus*		1/1	Manatee
Perissodactyla	Equidae	*Equus*	*caballus*	1/35	Domestic horse
		Equus	*burchelli*	0/1	Zebra
		Equus	*grevyi*	0/1	Zebra
	Rhinocerotidae	*Diceros*	*bicornis*	1/1	Black rhino

TABLE IV—*Concluded*

Order	Family	Genus	Species	No. positive / No. tested	Common name
Artiodactyla	Suidae	*Sus*	*scrofa*	23/23	Domestic pig
		Phacochoerus	*arthiopicus*	1/1	Warthog
	Hippopotamidae	*Hippopotamus*	*amphibius*	1/1	Hippopotamus
	Camelidae	*Lama*	*glama*	1/1	Llama
		Cametus	*dromedarius*	10/10	Camel
Artiodactyla	Cervidae	*Muntiacus*	*reevesii micrurus*	1/1	Muntjak
		Odocoileus	*virginianus*	0/11	White-tailed deer
		Alces	*alces*	0/17	Moose
	Giraffidae	*Giraffa*	*camelopardalis*	3/3	Giraffe
	Bovidae	*Strepsiceros*	*strepsiceros*	1/1	Greater kudu
		Tragelaphus	*scriptus*	1/1	Bushbuck
		Limnotragus	*spekii*	1/1	Sitatunga
		Taurotragus	*oryx*	1/1	Eland
		Bos	*taurus*	31/31	Domestic cattle
		Bison	*bonasus*	7/7	Aurochs
		Syncerus	*caffer*	1/1	Buffalo (Cape)
		Bison	*bison*	1/1	Bison
		Sylvicapra	*grimmia*	1/1	Duiker
				1/1	Duiker from Kaduna
		Kobus	*ellipsipryamnus*	1/1	Waterbuck
		Hippotragus	*equinus*	1/1	Roan antelope
		Hippotragus	*niger*	1/1	Sable antelope
		Oryx	*beisa*	1/1	Oryx
		Alcelaphus	*buselaphus cokii*	1/1	Hartebeest cokes
		Alcelaphus	*buselaphus jacksoni*	0/1	Hartebeest Jackson's
		Alcelaphus	*lichensteinii*	1/1	Hartebeest Lichenstein
		Ourebia	*ourebi*	0/1	Oribi
		Aepyceros	*melampus*	0/1	Impala
		Gazella	*thomsonii*	0/1	Thomson's gazelle
		Gazella	*granti*	0/1	Grant's gazelle
		Ovis	*aries*	30/30	Sheep
		Capra	*hircus*	33/37	Goat

protein of many other mammalian species. In the rabbit, on the other hand, many of the amino acid sequences, contained in other mammalian proteins of MuB1 type, are present; the animals are tolerant to this protein; large portions of it are excluded from immunogenicity, and the antibody shows a correspondingly high degree of specificity (see reference 20).

We have, so far, established that MuB1-negative mice do not possess an antigen which has a significant proportion of the determinants of the antigen MuB1. We turned next to an examination of the inheritance of MuB1.

Inheritance of MuB1.—A number of hybrids of inbred strains were tested and it was found that whenever one or both parents possessed the antigen, MuB1, the offspring also had this antigen. Only hybrids between parents which both lacked the antigen MuB1, such as AKR/J and DBA/2J, did not possess the antigen (Table V). In order to analyse the nature of the inheritance further, hybrids from one parent possessing MuB1 and another parent lacking this

antigen were backcrossed with an animal which lacked the antigen. The parents possessing the MuB1 antigen were of strains C57BL/6J and C57L/J. The parents lacking the antigen were of strain A/J. The offspring of these matings were crossed with A/J mice. Each of these two types of backcrosses was performed with male hybrids and female mice of A/J strain and with female hybrids crossed with male animals of A/J strain. In all cases the offspring of these matings were bled when they were 5 to 7 weeks old and were tested by double diffusion in agar for the presence or absence of MuB1 in their serum. Approximately half of the offspring of the backcrosses possessed the antigen MuB1. However, there were generally more MuB1-negative mice than would be ex-

TABLE V

Presence and Absence of MuB1 in Hybrids

Female parent		Male parent		MuB1 in hybrid, No. positive/No. tested
Strain	MuB1	Strain	MuB1	
AKR/J	−	DBA/2J	−	0/8
BALB/cJ	+	A/J	−	7/7
C3H/HeJ	+	DBA/2J	−	9/9
C3H/HeN	+	C57BL/6J	+	1/1
C57BL/HaOci	+	C3H/HeNe$_f$Oci	+	1/1
C57BL/6J	+	A/J	−	8/8
C57BL/6J	+	DBA/2J	−	7/7
C57BL/Ha	+	C3H$_f$/Ha	+	1/1
C57L/J	+	A/J	−	9/9

pected on the basis of unifactorial inheritance of MuB1 (Table VI). These differences, probably due to a low sensitivity of the test for MuB1, are statistically insignificant. It follows that MuB1 is a hereditary characteristic determined by a single dominant or codominant gene. There was no correlation between the sex of the hybrids and the presence or absence of MuB1 (Table VI).

We examined next whether there was a correlation between the locus of MuB1 and the inheritance of the previously described allotype of gamma globulin, MuA2. For this purpose we tested the serum of several inbred strains of mice by double diffusion against antiserum to MuB1 and to MuA2 (Table I). Next, the sera of offspring from a double backcross (C57BL/6J × A/J) × A/J were tested with antibody specific for MuA2 (Table VI). Tables I and VI summarize our findings, which indicate quite clearly that there is no genetic link between MuA2 and MuB1. The difference between the experimentally determined distribution and the distribution expected if there were no correlation is not significant ($0.5 > P > 0.25$) (Table VI).

Whereas the presence or absence of the antigen MuB1 is not correlated with

the sex of parents and offspring, the concentration of this antigen in male and female animals of the same age and strain is not identical.

A Sex-Associated Difference in the Concentration of MuB1.—Sex-associated differences in the concentration of various serum proteins have been reported in rats (25) and cattle (26) and recently in several instances in the mouse (27, 28). We have examined the concentration of MuB1 by the double diffusion dilution test in the serum of mice of strains BALB/cJ, BSVS/Sr, BUB/Wi, C58/J, CBA/J, DBA/1J, DBA/1JSn, DBA/LiA, DBA$_t$/Sp-D, MO/Ko, SJL/J, T6, and WH/Ht. The concentration of antigen MuB1 was found in all these to be lower in female than in male mice. The sera used in this test were obtained from animals of different ages, but the age of males and females of the same strain was always identical. The wide variation in the antigen concentration of animals of the same sex, but of different strain and age led us to examine by single diffusion the antigen concentration as a function of age. It was found that the content of MuB1 increases with age, most markedly in males, less in females.

TABLE VI

Inheritance of MuB1 and the Correlation between Its Incidence with Sex, MuA2, and Hemolytic Complement

Summary of χ^2-tests, backcrosses between male and female hybrids (C57L/J × A/J) (C57BL/6J × A/J) with A/J of opposite sex.

Tests for correlation between:	MuB1		Total tested	χ^2	P
	Present	Absent			
Observed and expected distribution of MuB1*	226 (244.5)	263 (244.5)	489	2.800	$0.1 > P > 0.05$
MuB1 and sex of the hybrids‡	♂ 115 (114.6) ♀ 111 (111.4)	♂ 133 (133.4) ♀ 130 (129.6)	489	0.0053	$0.95 > P > 0.9$
MuB1 and MuA2‡, §					
MuA2 present	42 (44.4)	53 (50.6)	182	0.510	$0.5 > P > 0.25$
MuA2 absent	43 (40.6)	44 (46.4)			
MuB1 and hemolytic complement (H.C.)‡					
H.C. present:	75 (38.7)	7 (43.3)	159	133.16	$P < 0.005$
H.C. absent:	0 (36.3)	77 (40.7)			

* Figures in brackets are based on the assumption of unifactorial inheritance of MuB1.

‡ Figures in brackets give the expected numbers calculated on the assumption that there is no correlation.

§ Hybrids (C57BL/6J × A/J) × A/J only.

The difference in the concentration between the sexes could be seen at all ages between 3 weeks and 6 months (Table VII).

Having dealt with the distribution, inheritance, and sex- and age-associated differences of the concentration of MuB1, we shall turn next to the function of this antigen.

Complement and MuB1.—The existence of the complete complement system in the mouse has long been in doubt, since the hemolytic test developed for demonstration of complement in guinea pig and man did not appear to give satisfactory results with complement of the mouse. However, in 1911 Ritz (29) established that the first component of complement was present in mice; Brown

TABLE VII

The Variation of Concentration of MuB1 in the Serum of Mice of Strain DBA/1J as a Function of Age and Sex

Age	Concentration of MuB1*	
	♂	♀
days		
180	1.0	0.444
56	0.476	0.444
49	0.455	—‡
28	0.444	0.408
21	0.408	0.392

* Concentration measured by single diffusion and expressed as a fraction of the concentration of MuB1 in the serum of male DBA/1J mice, 6 months of age.

‡ Not used.

(30) showed that C'3 and C'4 were also present, but Muschel and Muto's (31) experiment seemed to indicate that C'2 and C'3 were absent. However, McGhee (32) succeeded in demonstrating 1 to 2 units of hemolytic complement activity per ml, and refinements in the demonstration of the presence of hemolytic activity of mouse complement allowed Borsos and Cooper (33) to demonstrate clearly that C'1, C'4, C'2, and C'3 components of complement were all present in mouse serum. More recently, Rosenberg and Tachibana and Herzenberg *et al.* have demonstrated that some mice have an incomplete complement system, whereas others have a complete complement system (17), and they have designated the factor in question as Hc (10).

In order to explore the possibility that the antigen MuB1 might be connected with this factor, we compared mice for the presence of hemolytic complement and the presence of MuB1 in 24 inbred strains of mice. It was found that wherever MuB1 was present, a complete hemolytic complement system could be demonstrated (BALB/cJ, BDP/J, BUB/Bn, CBA/J, C57BL/Ha,

C57BL/6J, C57BR/cdJ, C57L/J, C58/J, MA/J, P/J, PL/J, SL/R1, SJL/J, SM/J, T6, 129/J) and wherever MuB1 was absent, a functioning hemolytic system could not be demonstrated (A/HeJ, AKR/J, CE/J, DBA/2J, DBA/2DeJ, RF/J, and SWR/J). We next tested for the presence and absence of the complement system in backcrosses of hybrids with one of the parent strains, and it will be seen from Table VI that there is reasonable correlation between the presence of MuB1 and a hemolytic complement system. Thus it may be concluded that the presence of MuB1 is a necessary prerequisite for the possession of a complete complement system.

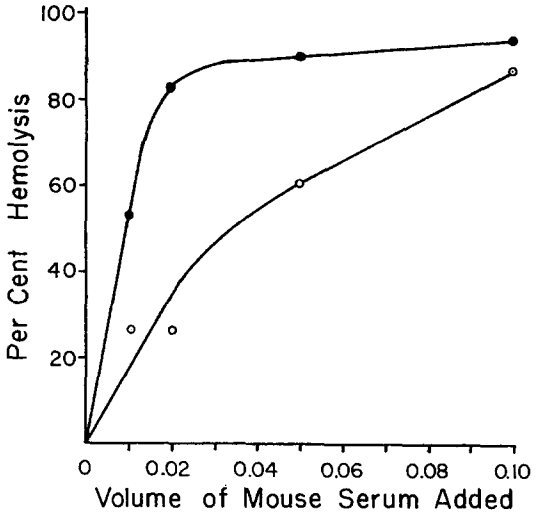

FIG. 2. Per cent hemolysis as a function of the volume of fresh serum added to sensitized red cells: The difference between the hemolytic effect of serum from male and from female mice, 6 weeks old, strain BALB/cJ. — ● —, serum from male mice;— ○ — serum from female mice.

To test this conclusion further, we compared the concentration of MuB1 by single diffusion in male and female mice of strain BALB/cJ and found that male individuals contained a higher concentration of the antigen MuB1 than did female individuals. We then measured hemolytic complement in male and female individuals of the same strain and found a greater number of hemolytic units in male than in female mice (Fig. 2). Similar results were obtained with other strains and it thus seemed that the hemolytic efficiency of the complete complement system of individuals of this strain could be correlated with the concentration of MuB1. However, the ratio of antigen in the serum of male and female mice of strain BALB/cJ was approximately 1.4 (see preceding section) and that of the hemolytic units was close to 5.0.

In the above section we referred to a "complete hemolytic complement system," implying that the complement is supplied entirely by the mouse serum. This may not, however, be the case, for the rabbit hemolysin is used at such a high concentration that components of rabbit complement may also be contributed in significant amounts. In fact, it is not at all clear whether the requirement for a high concentration of hemolysin is for the antibody itself or for antibody plus certain components of rabbit complement. In attempting to resolve this question we separated the macroglobulin fraction of the antiserum which contained the major part of the hemolytic activity (as judged by tests with guinea pig complement) and used it to test mouse complement. The concentration of the fraction was equivalent to a $\frac{1}{10}$ dilution of the unfractionated serum. No hemolysis occurred in this test, suggesting that the macroglobulin fraction of the hemolysin lacked some factor of different molecular weight which was present in the original rabbit serum and which was necessary for hemolytic activity of mouse complement. Clearly, much further work remains to be done in the direction of determining optimum conditions for the assay of mouse complement and the test used in our studies must be considered quite crude. Notwithstanding this limitation, the fact remains that the test as performed enabled us to separate mouse sera into positive and negative categories which correlated well with the presence and absence of MuB1.

Properties of MuB1 and of the Corresponding Antigen in Other Species.—If serum, containing MuB1 or the corresponding human antigen, is kept at 56°C for 60 minutes, the serum loses its reactivity with the antibody to MuB1. This loss in reactivity of the mouse antigen was examined by single diffusion in agar and it was found that 50 per cent of the reactivity was lost after 17 minutes at 56°C. Treatment with zymosan, hydrazine, or ammonia did not reduce the reactivity of the human and guinea pig antigen with MuB1 antibody. When mouse serum was subjected to gel filtration on sephadex G-200, by the method of Roskes and Thompson (19), the antigen MuB1 was located in the middle peak which contains the proteins of molecular weight of about 150,000.

The antigen MuB1 and the related antigen of all mammalian species tested, can be separated from serum by dialysis of serum against distilled water or by dilution of serum with 10 volumes of distilled water, saturated with CO_2 at +4°C. The antigen is found in the resulting precipitate. By redissolving the precipitate in a fraction of the original serum volume, the antigen MuB1 can be

FIG. 3. Immunoelectrophoretic analysis of mammalian antigens reacting with antibody to MuB1. The wells contained euglobulin fraction prepared from the sera of the following species of mammals (see Table IV): *A*, human; *B*, two-toed sloth; *C*, rabbit; *D*, groundhog; *E*, woodland deer mouse; *F*, mouse of strain C57L/J; *G*, rat; *H*, porcupine; *I*, sperm whale; *J*, racoon; *K*, skunk; *L*, mink; *M*, dog; *N*, lynx; *O*, goat; *P*, cattle. All channels contained antiserum to MuB1 from mice of strain A/HeJ which had been immunized with serum from mice of strain C57L/J.

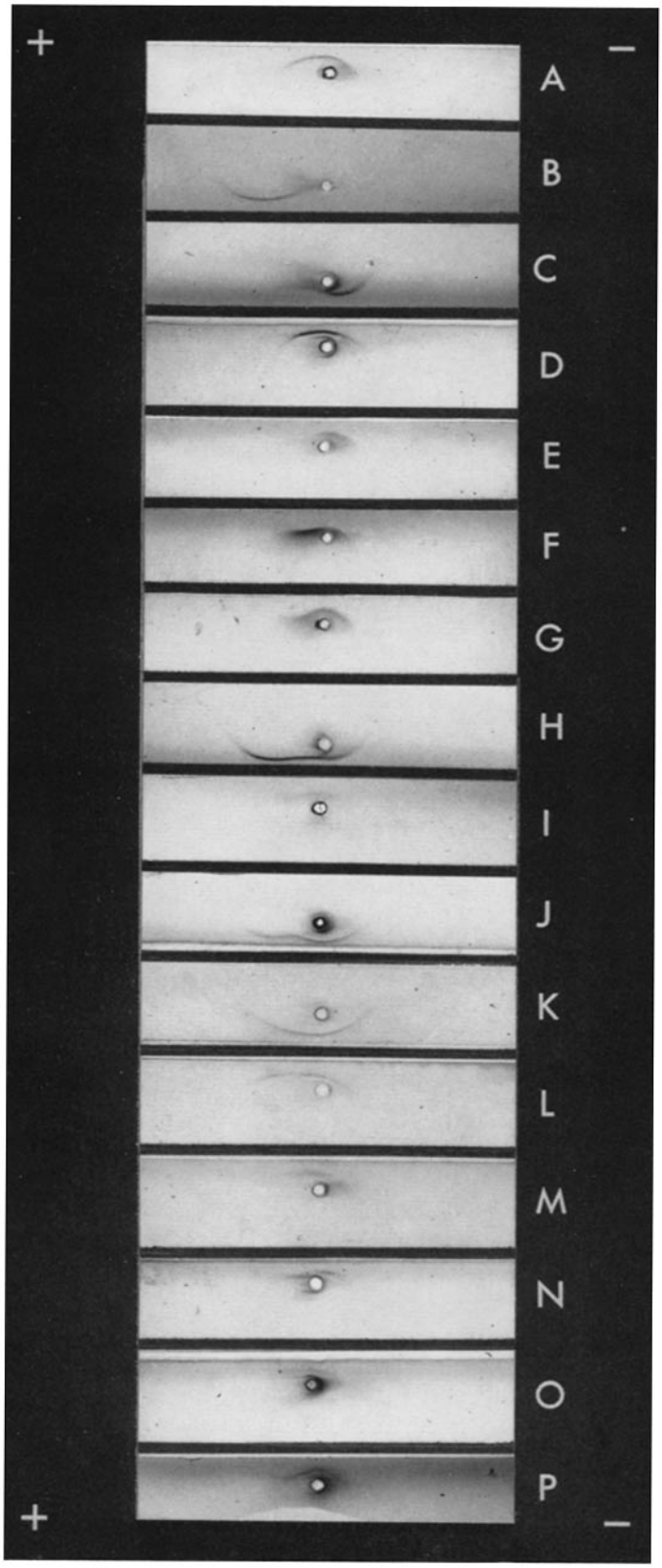

FIG. 3

concentrated. By the double diffusion-dilution method, 95 per cent of the guinea pig antigen present in serum was found in the euglobulin fraction and none in the concentrated pseudoglobulin fraction. The immunoelectrophoretic experiments to be described next were carried out with such concentrated eu-

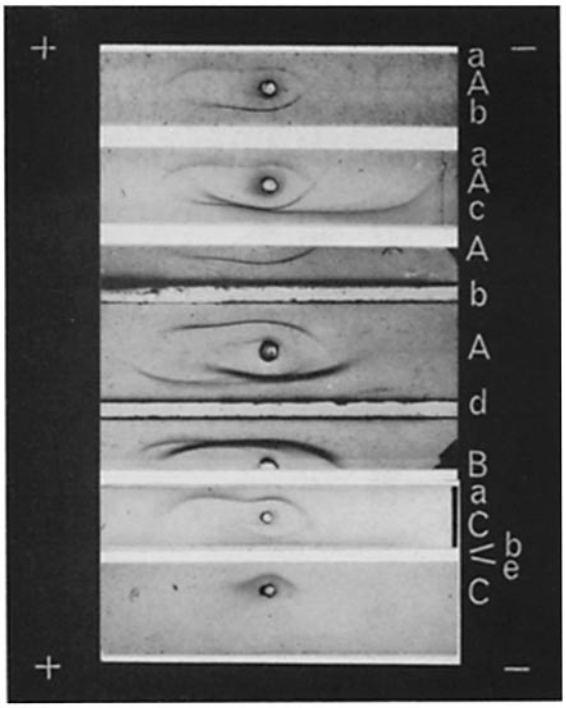

FIG. 4. A comparison of the mobility of some mouse and rat antigens reacting with antibody to MuB1 and with antibody to other serum proteins. The wells contained: *A*, euglobulin of mouse serum (DBA/1J); *B*, peritoneal exudates from myeloma mice; *C*, rat euglobulin. The channels contained: *a*, Antiserum to rat α_2-macroglobulin; *b*, Antiserum (rabbit) to MuB1 absorbed with a pseudoglobulin fraction of MuB1-positive mouse serum; *c*, unabsorbed antiserum (rabbit) to MuB1; *d*, antiserum (rabbit) to a mouse myeloma antigen; *e*, antiserum to MuB1 from mice of strain A/HeJ.

globulin solutions. The antigen obtained from mouse sera migrated as a single component. The mobilities of the antigens of most species were very similar, except for the antigen of the two-toed sloth (Fig. 3, *B*) which migrated faster and of the lynx (Fig. 3, *N*) and rabbit (Fig. 3, *C*) which migrated more slowly than the antigen of the other species examined. A heterogeneity in mobility was observed with the serum of some mammalian species; *i.e.*, porcupine (Fig. 3, *H*) and racoon (Fig. 3, *J*).

The mobility of the mouse antigen MuB1 was characterized by comparison

with the α_2-macroglobulin, shown by interaction with cross-reacting antibody to the rat macroglobulin, and by comparison with myeloma protein of the mouse. The mouse antigen MuB1 showed no reaction with either of these antibodies; its mobility was greater than that of the myeloma protein and close to that of α_2-macroglobulin (Fig. 4).

The mobility of the rat antigen, corresponding to MuB1, was found to be identical with the slower of the two components of rat α_2-macroglobulin (Fig. 4).

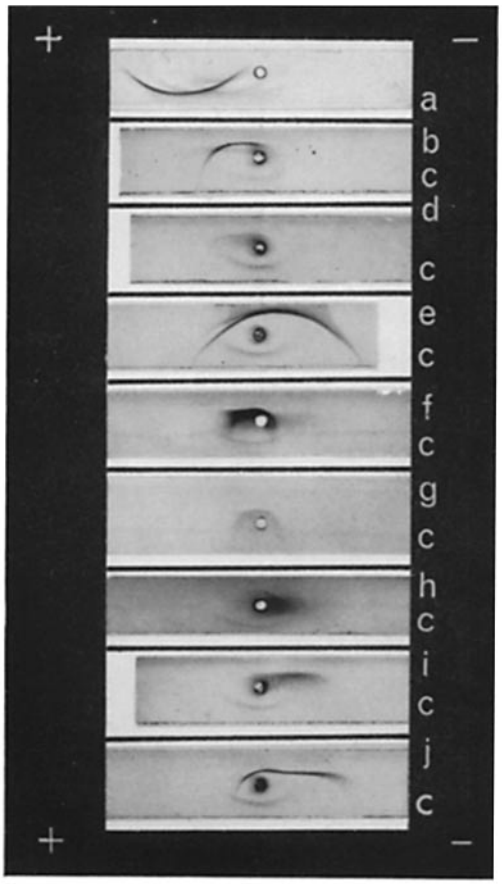

FIG. 5. The relative mobility of the human antigen reacting with mouse antibody to MuB1 and of other human serum proteins. All centre wells contain human serum euglobulins. Channels contain the antisera to the following human serum proteins: *a*, oroso mucoid; *b*, α_2-macroglobulin; *c*, mouse antiserum to MuB1; *d*, ceruloplasmin; *e*, transferrin; *f*, β-lipoprotein; *g*, β-lipoprotein (the slide was treated with Sudan IV); *h*, Ag(a+); *i*, β_2-A-globulin; *j*, β_2-M globulin.

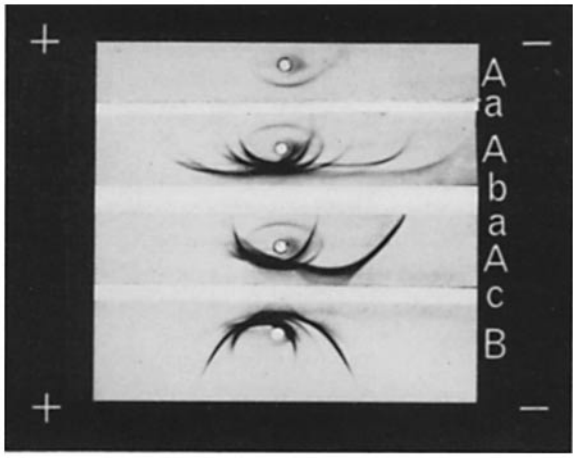

FIG. 6. The hydrazine stability of the human antigen reacting with MuB1 antiserum and of the human β_1-C component of the C'3 complex. The centre wells contain the following: *A*, human serum euglobulin; *B*, human serum euglobulin treated with hydrazine. The channels contain: *a*, mouse antiserum to MuB1; *b*, antiserum to C'1 esterase; *c*, antiserum to the β_1-C component of the C'3 complex.

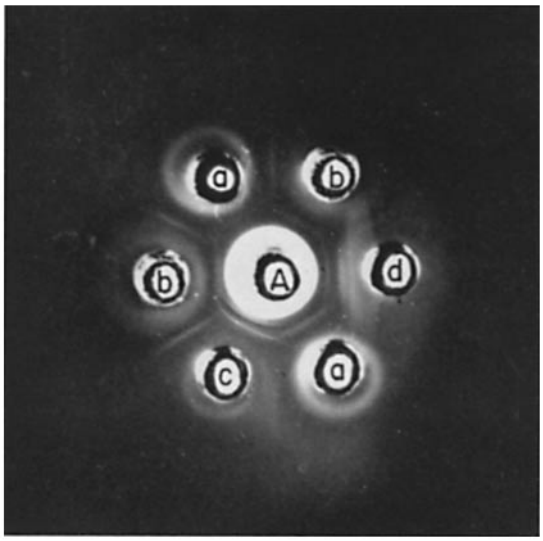

FIG. 7. The reaction between human serum and antibody to MuB1 and multispecific antibodies to various components of complement. Antisera to MuB1, C'1 esterase, and β_1-C all contain an antibody, reacting with the same antigen. The peripheral wells contain: *a*, antibody (rabbit) to S11 ($C'1_q$); *b*, antiserum (mouse) to MuB1; *c*, antiserum (rabbit) to C'1 esterase (diluted 1:2); *d*, antiserum to the β_1-C component of the C'3 complex. The centre well contains: *A*, human serum.

The mobility of the human antigen was compared with that of several defined protein components. It was found to migrate with a mobility close to that of beta lipoprotein and of Ag(a+) (13) (Fig. 5). Unlike beta lipoprotein, it was, however, not stained by Sudan IV and is not a lipoprotein. The reaction of MuB1 with human serum was also compared with that of antibody to S11, C'1 and β_1-C globulin (Fig. 6). The antibody to S11 reacts with two components of which one is no longer detectable when the euglobulin solution is heated. The heat-stable component has been identified with γ_1M (Christian, personal communication). The heat-labile component is the component of sedimentation constant S11 (11). The antigen reacting with MuB1 antibody does not show in double diffusion or immunoelectrophoresis any cross-reactivity with either γ_1M or the S11 component. It follows that the antibody to MuB1 is distinct from S11.

The most prominent component in the antiserum to human β_1-C globulin is directed against an antigen which migrates with two distinct mobilities before treatment with hydrazine and only with one after treatment with hydrazine. This, presumably, is β_1-C globulin. The other antigens, detected by the antibody, are not affected in their reactivity after this treatment. Amongst them is an antigen with which both the antiserum to β_1-C and the antiserum to C'1 react (Fig. 7). This antigen is identical with the antigen with which the antibody to MuB1 combines. It follows that anti-MuB1 reacts with an antigen other than β_1-C. The antigen with which the antibody to MuB1 and C'1 reacts loses its reactivity if human serum is heated for 1 hour at 56°C.

DISCUSSION

The antigen, designated MuB1 (8), is a heat-labile euglobulin, detected by an antibody raised in mouse or rabbit, and present in 61 per cent of inbred strains of mice tested. Related molecules can be demonstrated in a wide variety of mammalian species by means of the cross-reacting murine antibody to MuB1. The question, whether the product of a gene, allelic to that controlling the synthesis of MuB1, occurs in MuB1-negative mice, has been explored by examining the specificity of rabbit antibody to MuB1. The absence of such reactivity between this antibody and an antigen corresponding to MuB1 in MuB1-negative mice (see section on Status of MuB1-Negative Mice), indicates that MuB1-negative mice do not possess an antigen corresponding to MuB1 or that this antigen is very similar to a rabbit antigen, a most improbable contingency.

An independent, but less direct, indication of the absence of an antigen corresponding to MuB1 in MuB1-negative animals, may be deduced from the reaction of the mouse (A/HeJ) antibody with the antigen of many different mammals. This extensive reactivity is partly due to the high antibody content of the antiserum used. A much less potent murine antibody, obtained from a different strain (DBA/2J)-serum (DBA/1J) combination, showed a considerably reduced

reactivity with other mammals but yet more reactivity than the rabbit antiserum which contained at least twice as much antibody as judged by its reaction with a mouse serum (DBA/1J). It is clear, therefore, that specificity rather than potency is the decisive difference in the cross-reactivity of murine and rabbit antibody to MuB1. We have previously suggested (1, 34) that the specificity of the immune response, in general, depends largely on tolerance to autologous proteins.

It is convenient, in this context, to consider the determinants of a protein in terms of their evolutionary stability. Accordingly, one might postulate mammalian determinants, which remain unchanged throughout all the orders of *Mammalia*, order determinants (say *Rodentia* determinants), which remain unchanged during the evolution of the order, and finally species determinants which remain unchanged during the evolution of the species. Where two individuals of the same species differ in an allotypic determinant only, both individuals would possess, in their circulation, general mammalian determinants and also order and species determinants. When an animal is tolerant to the determinants of an autologous protein all the determinants of this protein are excluded from immunogenicity in this individual. Thus one would expect that antibody against a serum protein, raised in an animal of the same species as that of the donor of the serum protein, would be highly specific in the sense that it would be directed only to the particular determinant by which individuals of this species differ from each other. Such an antibody would not react with the serum of animals of a different species (20). The reactivity of antibody to MuB1 is, therefore, of considerable interest in not conforming to this general rule. This isologous antibody reacts not only with the serum of other rodents but also with the serum of many distantly related mammalian species (see Table IV).

The above exception to the general rule of narrow specificity, when donor of antigen is closely related to donor of antibody, could find a ready explanation, namely that the animals which do not possess the molecule, MuB1, completely lack all its determinants.

There are thus several independent lines of evidence which lead us to the conclusion that some mice possess an antigen MuB1 and that other mice lack this antigen and any molecule structurally related to it.

There is excellent agreement between the absence of MuB1 and the absence of hemolytic complement in inbred strains of mice. In backcross experiments, too, a highly significant correlation between these two properties was found (Table VI), but in the latter instance there was not complete agreement between the two tests. This may be attributable to relatively low concentrations of MuB1 and of complement in some of the backcrosses, possibly because of a single dosage effect which may result in a lower concentration of antigen in heterozygous than in homozygous animals. Also, the concentration of antigen and of complement may be such that the tests for their detection (Table VI)

are being used at the limit of their sensitivity so that slight variations in concentration may be the cause of failure in detection.

A relation between the antigen MuB1 and the hemolytic system may also be deduced from the correlation between quantity of hemolytic complement and the quantity of MuB1 in male and female mice, although it must be stressed that serum proteins, other than MuB1, show sex-associated differences (27, 28), and that the relative quantities of antigen MuB1 and of hemolytic complement units in the sera of the two sexes are not identical.

As judged by strain distribution, similar except for CBA and AKR mice, and fusion of zones in double diffusion, MuB1 (8, 9) appears to be closely related to the antigen described by Erickson, Tachibana, Herzenberg, and Rosenberg (35), with whom we have exchanged antisera. It must be stressed, however, that the identity of these antigens (Cinader and Dubiski, 1963, reference 8; Erickson, Tachibana, Herzenberg and Rosenberg, 1964, reference 35) with the complement factor Hc defined by Herzenberg *et al.* in 1963 (10), has, so far, not been established by direct test.

Two recent papers have dealt with complement deficiency in mice and its correlation with absence of an antigen which could be detected in complement-containing mice by double diffusion in agar (35, 36). Our findings (9) are in complete agreement with the inheritance reported for the antigen, linked with complement deficiency (35), and with the report of a sex dependence of hemolytic efficiency (36).

Terry, Borsos, and Rapp (36) have suggested that the complement deficiency of mice is attributable to lack of C'3. The properties of MuB1, reported in the present paper, are compatible with this conclusion, in view of the absence of C'3 components (37) and MuB1 (see Table IV) in the horse, and of the heat lability and hydrazine sensitivity of C'3d (37).

SUMMARY

An antigen, MuB1, present in the sera of some mice, can elicit a precipitating antibody in certain other strains of mice. An antibody to the antigen MuB1 can also be elicited in rabbits. 99 strains and substrains of inbred mice were tested for the presence of MuB1; the antigen was found in the sera of 44 strains (61 per cent) and 14 DBA substrains (52 per cent). Evidence is presented indicating that mice lacking MuB1 do not make a modified antigen, corresponding to MuB1, but are genetically deficient in synthetic ability at this site.

By reaction with antibody to MuB1 an antigen corresponding to MuB1 was found in 13 of the 15 orders of mammals, and in 63 of 85 mammalian species tested, including man and guinea pig.

The quantity of the antigen MuB1 is always greater in the serum of male than in the serum of female mice. The concentration of MuB1 increases with age; this increase is more marked in male than in female mice.

By means of backcross experiments it was shown that the inheritance of MuB1 is unifactorial, is independent of the inheritance of the gamma globulin allotype MuA2, and is qualitatively independent of the sex of the parents.

The antigen MuB1 is found in the euglobulin fraction of serum; it loses its ability to precipitate with antibody after heating at 56°C, but not after treatment with ammonia or hydrazine. By gel filtration, MuB1 is separated with a fraction containing molecules of molecular weight $\approx$ 150,000.

An empirical correlation was observed between the presence or absence of MuB1 in the sera from inbred mice and the presence or absence of hemolytic complement (Hc), as measured by a test using a high concentration of rabbit hemolysin. In backcross experiments also, a correlation between hemolytic complement and the presence of MuB1 was demonstrated. As with MuB1, male mice had a higher hemolytic complement level than females. The particular component of complement which may be identical with MuB1 has not been identified.

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BIBLIOGRAPHY

1. Cinader, B., and Dubiski, S., The effect on immunogenicity of acquired immunological tolerance, *Colloq. Internat. Centre Nat. Recherche Sc.*, 1963, **116,** 255.
2. Oudin, J., L' "Allotypie" de certains antigènes protéidiques du sérum, *Compt. Rend. Acad. Sc.*, 1956, **242,** 2606.
3. Dray, S., Dubiski, S., Kelus, A., Lennox, E. S., and Oudin, J., A notation for allotypy, *Nature*, 1962, **195,** 785.
4. Stefanini, M., and Dameshek, W., Hemorrhagic Disorders, New York, Grune and Stratton, Inc., 2nd edition, 1962.
5. Biggs, R., and MacFarlane, R. G., Human Blood Coagulation and Its Disorders, Oxford, England, Blackwell Scientific Publications, 3rd edition, 1962.
6. Harris, H., Human Biochemical Genetics, London, Cambridge University Press, 1963.
7. Hsia, D. Y., Inborn Errors of Metabolism, Chicago, Year Book Publishers, 1959.
8. Cinader, B., and Dubiski, S., An alpha-globulin allotype in the mouse (MuB1), *Nature*, 1963, **200,** 781.
9. Cinader, B., and Dubiski, S., Effect of autologous protein on the specificity of the antibody response: mouse and rabbit antibody to MuB1, *Nature*, 1964, **202,** 102.
10. Herzenberg, L. A., Tachibana, D. K., Herzenberg, L. A., and Rosenberg, L. T., A gene locus concerned with hemolytic complement in *Mus musculus*, *Genetics*, 1963, **48,** 711.
11. Lepow, I. H., Studies on antibodies to human C'1-esterase, *Ann. New York Acad. Sc.*, 1963, **103,** 829.
12. Morse, J. H., and Christian, C. L., Immunological studies of the 11S protein component of the human complement system, *J. Exp. Med.*, 1964, **119,** 195.
13. Blumberg, B. S., Iso-antibodies in humans against inherited serum low density beta-lipoproteins, the Ag system, *Ann. New York Acad. Sc.*, 1963, **103,** 1052.
14. Oudin, J. B., Specific precipitation in gels and its application to immunochemical analysis, *Methods Med. Research*, 1952, **5,** 335.
15. Gilder, R. S., Dark ground photography of precipitation bands, *Med. and Biol. Illus.*, 1963, **13,** 231.
16. Kabat, E. A., Kabat and Mayer's *Experimental Immunochemistry*, Springfield, Illinois, Charles C Thomas, 2nd edition, 1961, 241.
17. Rosenberg, L. T., and Tachibana, D. K., Activity of mouse complement, *J. Immunol.*, 1962, **89,** 861.
18. Darter, L. A. D., Procedure for production of anti-sheep hemolysin, *J. Lab. and Clin. Med.*, 1953, **41,** 653.
19. Roskes, S. D., and Thompson, T. E., A simple molecular sieve technique for detecting macroglobulinemia, *Clin. Chim. Acta*, 1963, **8,** 489.
20. Cinader, B., Perspectives and prospects of immunotherapy. Autoantibodies and acquired immunological tolerance, *Canad. Cancer Conf.*, 1963, **5,** 279.
21. Dubiski, S., and Cinader, B., A new allotypic specificity in the mouse (MuA2), *Nature*, 1963, **197,** 705.
22. Dubiski, S., and Cinader, B., A new allotypic specificity in the mouse (MuA2), *Canad. J. Biochem. and Physiol.*, 1963, **41,** 1311.

23. Wunderlich, J., and Herzenberg, L. A., Genetics of a gamma globulin isoantigen (allotype) in the mouse, *Proc. Nat. Acad. Sc.*, 1963, **49,** 592.
24. Rother, U., and Rother, K., Über einen angebornenen Komplement-Defekt bei Kaninchen, *Z. Immunitätsforsch.*, 1961, **121,** 224.
25. Heim, W. G., Sex and sex-cycle differences in rat serum protein concentrations, *Am. J. Physiol.*, 1959, **102,** 704.
26. Dimopoullos, G. T., Schrader, G. T., and Fletcher, B. H., Electrophoretic studies of bovine serum. IV. Differences in serum glycoproteins due to age and sex, *Proc. Soc. Exp. Biol. and Med.*, 1959, **102,** 704.
27. Cons, J. M., and Glass, L. E., Electrophoresis of serum proteins and selected enzymes in males, non-pregnant, pregnant, and lactating female mice, *Proc. Soc. Exp. Biol. and Med.*, 1963, **113,** 893.
28. Espinosa, E., Canelo, E., Bravo, M., and Gonzales, O., Sex-associated differences in serum proteins of mice, *Science*, 1964, **144,** 417.
29. Ritz, H., Über antikörperbildung und Anaphylaxie bei weiben Mäusen, *Z. Immunitätsforsch,. Orig.*, 1911, **9,** 321.
30. Brown, G. C., Complementary activity of mouse-serum, *J. Immunol.*, 1943, **46,** 319.
31. Muschel, L. H., and Muto, T., Bacterial reaction of mouse serum, *Science*, 1956, **123,** 62.
32. McGhee, R. B., Presence of complement in serum of the mouse, *Proc. Soc. Exp. Biol. and Med.*, 1952, **80,** 419.
33. Borsos, T., and Cooper, M., On the hemolytic activity of mouse complement, *Proc. Soc. Exp. Biol. and Med.*, 1961, **107,** 227.
34. Cinader, B., Specificity and inheritance of antibody response: A possible steering mechanism, *Nature*, 1960, **188,** 619.
35. Erickson, R. P., Tachibana, D. K., Herzenberg, L. A., and Rosenberg, L. T., A single gene controlling hemolytic complement and a serum antigen in the mouse, *J. Immunol.*, 1964, **92,** 611.
36. Terry, W. D., Borsos, T., and Rapp, H. J., Differences in serum complement activity among inbred strains of mice, *J. Immunol.*, 1964, **92,** 576.
37. Linscott, W. D., and Nishioka, K., Components of guinea pig complement. II. Separation of serum fractions essential for immune hemolysis, *J. Exp. Med.*, 1963, **118,** 795.