MOUSE COMPLEMENT: THE EFFECT OF SEX HORMONES AND CASTRATION ON TWO OF THE LATE-ACTING COMPONENTS*

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(Received for publication 21 November 1966)

By use of intermediate cell products of immune hemolysis made with guinea pig complement components, Borsos and Cooper demonstrated the presence of C'1, C'2, C'4, and C'3 complex in the sera of CFW mice, and laid the foundation for the measurement of mouse C' (C'^m) components (1). Rosenberg and Tachibana used an improved test for the titration of whole C'^m activity and found marked differences in hemolytic complement activity among inbred strains of mice (2). Herzenberg et al. (3) identified a euglobulin, hc^1, in the sera of mice whose blood contained hemolytic C'^m (C'^m-competent mice). Sera of C'^m-incompetent mice lacked this protein (3). Erickson et al. subsequently showed that the presence of C'^m activity and the protein hc^1 always segregated together and were determined by a single gene (4). Cinader, Dubiski, and Wardlaw (5), and Urbach and Cinader (6) have investigated a similar, if not identical, protein which they called MuB1. In C'^m-competent strains of mice they noted a higher concentration of MuB1 in the sera of adult males than in the sera of adult females. Nilsson and Müller-Eberhard used gel diffusion techniques to show that this genetically determined component is analogous to human C'5 (7).

Terry et al. studied the C'1, C'2, C'4, and C'3 complex activity of the sera of several strains of mice (8). The C'3 complex will be referred to as the late-acting components or C'^m-EDTA. Component studies indicated that inter- and intra-strain differences in C'^m activity were not due to variation of C'1, C'4, or C'2 activity. The genetically deficient strains, however, contained no measurable C'^m-EDTA activity. The sera of males of C'^m-competent strains had about 10 times as much C'^m-EDTA activity as the sera of females of the same strains (8). It thus seemed that both the genetic deficiency and the re-

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duced C'^m activity of the sera of females were due to a deficiency of at least one of the components acting after C'1, C'2, and C'4.

The study of genetic and hormonal control of complement may lead to a better understanding of the role of complement in a variety of biological processes (6, 9, 10). Accordingly, we studied the effect of castration and the in vivo effect of exogenous testosterone and estradiol upon mouse complement. The results of this study indicate that the serum content of at least two of the late-acting complement components, tentatively identified as C'5 and C'6, is affected by castration and by these hormones. The course of homograft rejection in B10D2 "old line" male mice rendered doubly deficient by castration, was identical to that of control mice.

Materials and Methods

Mice.—Balb/c mice were obtained from the Animal Production Section of the National Institutes of Health. B10D2-Old/SnJ (B10D2 "old line") and B10D2-New/SnJ (B10D2 "new line") mice were obtained from the Jackson Laboratory, Bar Harbor, Maine; these strains are coisogenic, differing only in that the "old line" is C'-deficient, the "new line," C'-competent.

Mouse Serum.—Mice were anesthetized with pentobarbital administered intraperitoneally, after which they were bled and killed by heart puncture. The fresh blood was placed in a centrifuge tube stored in an ice bath. Immediately after formation of the clot, the serum was collected and titrations were performed within 2–3 hr after the blood was obtained. Mice were used only for one bleeding.

Materials.—Veronal-buffered saline (VBS), sheep erythrocytes (E), and rabbit antibody to boiled stromas of sheep erythrocytes (A) were prepared as described in reference 11 (p. 149–150). Techniques for the preparation of cellular intermediate products consisting of E, A, and guinea pig (gp) C' components are given in reference 11 (p. 200–201). The preparation of VBS-EDTA (made by mixing 1 volume of 0.10 M Na_3 ethylenediaminetetraacetate, pH 7.5–7.6, with 9 volumes of VBS lacking Ca^{++} and Mg^{++}) and of C'-gp-EDTA are described in reference 12.

Hormones.—Aqueous testosterone (Oreton), given in the dosage of 2.5 mg per mouse, and estradiol benzoate in sesame oil (Progynon), given in the dosage 0.1 mg per mouse, were supplied by the Schering Co., Bloomfield, N. J. Controls were given Oreton vehicle (supplied by Schering) or sesame oil.

Complement Titrations.—

Whole complement: Sensitized cells (EA) were prepared by mixing a volume of E (1×10^9 cells/ml) with an equal volume of A. The diluent for both E and the A was VBS-EDTA buffer. The amount of antiserum used was 5–10 times the concentration of antiserum used to produce optimally sensitized cells for guinea pig C' titration. The sensitized cells (EA) were washed three times in VBS and brought to a concentration of 1×10^8 cells/ml in VBS. 0.2 ml portions of EA, containing 2×10^7 cells, were mixed with 1.0 ml of serial dilutions of mouse serum, and the mixtures were incubated at 37°C for 60 min with gentle mechanical agitation. About 4 ml of cold VBS were then added and the cells were collected by centrifugation. The supernatant fluids were discarded, the sedimented cells were lysed by addition of 3.0 ml distilled water, and the optical density of each lysate was measured at a wavelength of 412 mμ. Titers are expressed as the reciprocal of the dilution of mouse serum giving 50% lysis (C'H50) and were calculated by means of the von Krogh transformation (reference 11, p. 136).

C'1: C'1 activity was estimated by the ability of mouse serum to convert $EAC'4^{gp}$ to

EAC'1a^{m}, 4^{gp}. 0.5 ml portions of EAC'4^{gp} (1.5×10^8 cells/ml) were mixed with 0.5 ml of a dilution (from 1/10,000 to 1/80,000) of mouse serum and the mixtures were incubated for 15 min at 30°C. 0.5 ml of purified C'2^{gp} containing 100–200 effective molecules of C'2^{gp}/EAC'4^{gp} in VB-saline-sucrose ($\mu = 0.009$) was added to each sample and the mixtures were incubated for 10 min at 30°C to convert EAC'1a^{m}, 4^{gp} to EAC'1a^{m}, 4^{gp}, 2a^{gp}. 4 ml of a 1/50 dilution of guinea pig serum in 0.01 M EDTA (C'^{gp} EDTA 1/50) were added to convert EAC'1a^{m}, 4^{gp}, 2a^{gp} to E*. The mixtures were incubated at 37°C for 90 min, and the extent of lysis was determined spectrophotometrically. Titers are expressed as the dilution of mouse serum giving 63% lysis (C'1H63).

C'2: To estimate C'2 activity, sera were assayed for their ability to convert EAC'1a^{gp}, 4^{gp} to EAC'1a^{gp}, 4^{gp}, 2a^{m}. At zero time, a volume of EAC'1a^{gp}, 4^{gp} (1.5×10^8 cells/ml) was mixed with an equal volume of mouse serum diluted 1/400 in VBS and the mixture was incubated at 30°C with gentle mechanical agitation. At convenient times 0.5 ml samples of the mixture were withdrawn, mixed with 4.0 ml of C'^{gp}-EDTA 1/50, and incubated at 37°C for 90 min to convert EAC'4^{gp}, 2a^{m} to E*. Maximum generation of EAC'4^{gp}, 2a^{m} was achieved at 5 min ($t_{max} = 5$ min). Sera were compared on the basis of the fraction of cells lysable at t_{max} by C'^{gp}-EDTA.

C'4: Estimation of the C'4 content of mouse serum was based on the ability of the serum to generate sites in the state SAC'1a^{m}, 4^{m}, 2a^{m} from SA. The validity of this method rests on the observation that all mouse sera tested thus far contain similar amounts of C'1 and of C'2. Highly sensitized EA were made as in the method for whole C' titration. A volume of mouse serum, diluted 1/75, was incubated with an equal volume of EA (1.5×10^8 cells/ml) at 30°C with frequent mechanical agitation. 0.5 ml samples were withdrawn at convenient times and mixed with 4.0 ml of C'^{gp}-EDTA 1/50. Incubation and determination of lysis were performed as in the preceding section. Maximum generation of EAC'1a^{m}, 4^{m}, 2a^{m} occurred at 2 min ($t_{max} = 2$ min).

C'^{m}-EDTA: C'^{m}-EDTA activity of mouse serum was determined by its ability to generate E* from EAC'4^{gp}, 2a^{gp} in the presence of EDTA. The intermediate product EAC'1a^{gp}, 4^{gp} (1×10^9 cells/ml) was mixed with 2 volumes of purified C'2^{gp} and incubated at 30°C for 7 min. A large volume of ice cold 0.01 M EDTA was added and the cells were collected by centrifugation in the cold. The supernatant fluid was discarded and the cells were resuspended in 0.01 M EDTA to a concentration of 1.5×10^8 cells/ml and maintained at 0°C. For titration, 0.2 ml aliquots of EAC'4^{gp}, 2a^{gp} were placed in 13 × 100 mm test tubes at 0°C. To these were added 0.2 ml of serial dilutions of mouse serum in 0.01 M EDTA. The reaction mixtures were incubated at 37°C for 90 min with frequent agitation. About 3 ml of cold 0.01 M EDTA were added and the cells were collected by centrifugation. The extent of lysis was determined as described in the method for whole C' titration. Titers are expressed as the reciprocal of the dilution of mouse serum causing 50% lysis (C'^{m}-EDTA H50). Different lots of EAC'4^{gp}, 2a^{gp} vary in their susceptibility to lysis by C'-EDTA. Therefore, all titers were corrected to that of a standard lot of pooled guinea pig serum. In some experiments where high concentrations of mouse serum were used, the mouse serum was diluted in VBS and the EAC'4^{gp}, 2a^{gp} were suspended in 0.02 M EDTA to give a final concentration of 0.01 M EDTA in the reaction mixture.

Titration of C'5 and C'6: 0.2 ml of serial dilutions of test serum were mixed with a constant amount of appropriate reagent (see Results). The final dilution of reagent was the same in each reaction mixture. The C'5 or C'6 titer was calculated from the fraction of cells lysed in each mixture. Titers are expressed as reciprocal of dilution of serum giving 50% lysis. All calculations were based on the final dilution of test serum before addition of indicator cells. In contrast to C'^{m} assays, no attempt was made to correct for the day to day variation in EAC'4^{gp}, 2a^{gp} suspectibility to lysis.

C'6-Deficient Rabbit Serum.—Individual and pooled serum of C'6-deficient rabbits was obtained through the kindness of Dr. K. Rother and Dr. U. Rother (13).

RESULTS

Effect of Castration on the Activity of Serum C' Components.—Weanling and adult Balb/c mice of both sexes were castrated. Oophorectomies were

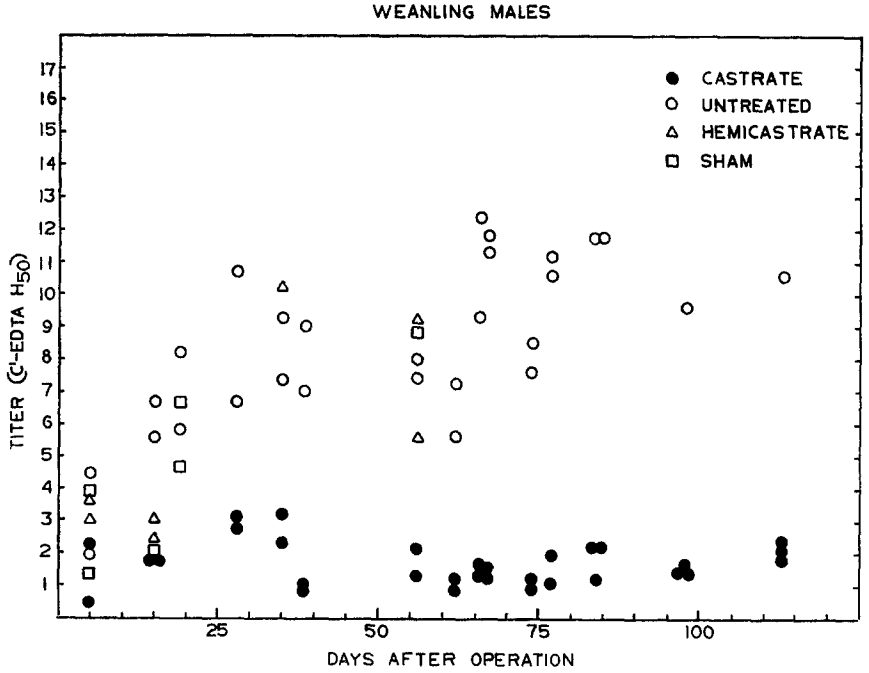

FIG. 1. The effect of castration on C'^{m}-EDTA activity of male mouse sera. Operation was performed on approximately 2-month-old mice. Each symbol represents the results of a single C'^{m}-EDTA titration of the serum from an individual mouse. (Reproduced by permission of the American Association for the Advancement of Science from: Weintraub, R. M., W. H. Churchill, Jr., C. Crisler, H. J. Rapp, and T. Borsos. 1966. Mouse complement: Influence of sex hormones on its activity. *Science.***152:**783.)

performed through posterior flank incision; orchiectomies, through a single scrotal incision. Controls consisted of mice from which only one gonad was removed, mice which were sham castrated, and untreated mice. At intervals after castration, the hemolytic activity of the late-acting complement components (C'^{m}-EDTA) of individual mice was assayed. As the weanling mice matured, there was a rise in C'^{m}-EDTA activity of the sera of control male animals. The C'^{m}-EDTA activity of the sera of castrated male mice stayed at a low level throughout the course of the experiment (Fig. 1). By 1 wk after castra-

tion, the sera of adult male mice contained significantly less C'^{m}-EDTA activity than those of the controls. Like the sera of castrated weanling males, the C'^{m}-EDTA activity of sera of castrated adult males remained, throughout the period of observation (4 months) at a low level of activity similar to that normally found in sera of control adult females. The C'^{m}-EDTA activity of sera from castrated females (weanling or adult) was slightly but significantly increased after 60 days following castration. There was no correlation between C'^{m}-EDTA activity and the weights of the mice at the time of bleeding.

The activities of C'1, C'2, and C'4 were determined in normal and castrated male mice. The data in Table I show that castration has no significant effect on the activities of these components. The low C'^{m}-EDTA activity of castrated

TABLE I

C'1, C'2, and C'4, Activities of the Sera of Normal and Castrated Male Balb/c Mice

Mice	C'1 (C'1H63)	C'4 Generation of EAC' $1^{m}4^{m}2^{m}$ at t_{max} (% lysis)	C'2 generation of EAC'$1a^{sp}4^{sp}2^{m}$ at t_{max} (% lysis)
Normal male	14,300	34.4	12.3
	15,400	29.3	15.1
	16,200		15.2
Castrated male	13,600	19.3	8.1
	7,600	36.2	9.4
	11,100		14.5
	16,200		

See Materials and Methods for experimental detail.

male and normal female mouse serum could be due to the presence of an inhibitor. Evidence against this hypothesis was obtained by the observation that the C'^{m}-EDTA activity of the serum of normal Balb/c males was not inhibited when this serum was mixed with increasing concentrations of the sera of either Balb/c female or castrated Balb/c male mice (Table II).

Effect, In Vivo, of Exogenous Testosterone and Estradiol on C'^{m}-EDTA Activity.—Testosterone and estradiol were administered to castrated and normal mice of both sexes. 4 days after a single injection of testosterone there was an increase in the C'^{m}-EDTA activity of the sera of castrated male mice. By the 8th day the level of C'^{m}-EDTA activity reached one-half that of normal male adults, but by the 17th day C'^{m}-EDTA activity fell to levels observed before hormone treatment.

Normal and castrated male and female mice were given weekly injections of testosterone. The C'^{m}-EDTA activity of the sera of all these mice was increased. The sera of a similar group of animals receiving weekly injections of estradiol instead of testosterone had reduced C'^{m}-EDTA activity (Table III).

TABLE II

Test for an Inhibitor in the Sera of Normal Female and Castrated Male Balb/c Mice

Serum 1	Serum 2	Lysis
		%
M 1/5	F	
	1/1	56
	1/2	57
	1/4	58
	1/8	55
	CM	
	1/1	60
	1/2	60
	1/4	57
	1/8	53
	Control (0.01 M EDTA)	46
		48
F 1/1	Control (0.01 M EDTA)	22
CM 1/1	Control (0.01 M EDTA)	27

A volume of serum 1 at the dilutions shown was mixed with an equal volume of serum 2 at the dilutions shown. Each mixture was tested for C'^{m}-EDTA hemolytic activity.

M: Normal male; F: normal female; and CM: castrated male.

TABLE III

C'^{m}-EDTA Titers of Serum of Mice Treated with Testosterone or Estradiol for 6–8 wk

Mice	No. of mice	Titer (C'^{m}-EDTA H50)		No. of mice	Titer (C'^{m}-EDTA H50)	
		Average	Range		Average	Range
		Testosterone			Control	
M	6	19.0	(13.2–21.0)	6	8.6	(5.0–13.5)
CM	6	13.0	(8.8–17.3)	6	3.0	(2.1–4.1)
F	6	8.8	(6.2–14.3)	6	2.1	(1.8–2.4)
		Estradiol			Control	
M	4	3.9	(2.0–6.2)	4	10.0	(7.8–13.0)
CF	4	1.8	(1.5–2.0)	4	1.9	(1.5–2.2)

M: normal male; CM: castrated male; F: normal female; and CF: castrated female.

Control mice were given either testosterone vehicle or sesame oil, neither of which affected C'^{m}-EDTA activity. In vitro treatment of sera with these hormones had no effect on the activity of the late-acting components.

Assay of C'5 in Mouse Serum.—Assays of C'5 were based on the ability of

samples to supply C'5 to the sera of B10D2 "old line" male mice, which lack this component (7). The adequacy of this serum as a reagent for measuring C'5 depends on the assumption that this serum lacks only C'5 and on its ability to furnish all other components at concentrations which do not limit the reaction. To test for this requirement, samples were assayed for C'5 content with three different high concentrations of reagent serum. The data in Table IV show that C'5 content titers were independent of the concentration of reagent.

Data were also plotted according to the equation for a one step reaction (14). Data plotted according to this equation will yield a straight line if a single

TABLE IV

Assay of C'5 in the Sera of Male and Female Balb/c Mice Using Different Concentrations of B10 D2 "Old Line" Male Serum as Reagent

Test serum	Final dilution* of B10D2 "old line" male serum reagent	Titer (C'5 H50)
Balb/c M	1/2	12.8
	1/3	13.6
	1/4	13.0
Balb/c F	1/2	5.4
	1/3	5.5
	1/4	6.1

M: Normal male; and F: normal female.

* Before addition of indicator cells. See Materials and Methods for experimental detail. C'^{m}-EDTA H50 of test serum alone: M = 7.7; and F = 1.38.

complement component is the independent variable, and all other components are supplied either in constant amount or in excess (Fig. 2). The straight lines representing mixtures of reagent serum and test serum provide evidence in favor of a one step reaction. This was interpreted to mean that the assay was a measure of a single molecular species, C'5.

Assay of C'6 in Mouse Serum.—Neither rabbit serum lacking C'6 (13) nor mouse serum lacking C'5 is hemolytically active. A mixture of these two sera has hemolytic activity. Consequently the serum of "old line" mice must contain at least C'6. We used the C'6-deficient rabbit serum as a reagent to assay C'6 activity in mouse sera. The C'6 titers of sera from "old line" females, B10D2 "new line" females, and Balb/c females were less than ¼ of the C'6 titer of sera from male mice of these strains.

The C'6-deficient rabbit serum, however, is not an ideal reagent for the assay of mouse C'6 because it inhibits the C'^{m}-EDTA activity of sera from Balb/c mice (Table V). This observation is consistent with the possibility that C'6-

deficient rabbit serum contains a factor or factors which inhibit the hemolytic activity of mouse serum. 13 pools of C'6-deficient rabbit serum were tested and

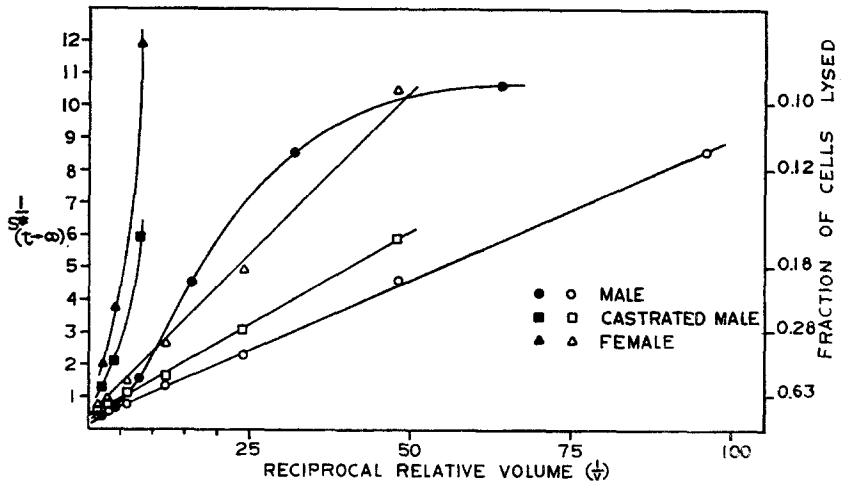

FIG. 2. Assay of C'5 in male, female, and castrated male Balb/c mice with pooled serum from B10D2 "old line" mice as a reagent, plotted according to the equation for a one step reaction. Open symbols: mixtures containing reagent serum at a final dilution of ⅓, plus test serum. Closed symbols: test serum alone. Similar results were obtained with reagent diluted ½ and ¼. Reagent serum alone caused no lysis.

TABLE V

Inhibition of C'^{m}-EDTA Activity by C'6-Deficient Rabbit Serum

Test serum	Reagent	C'^{m}-EDTA titer
Balb/c Male	C'6-deficient rabbit serum	6.8
" "	Control without rabbit serum	35.6
" Female	C'6-deficient rabbit serum	1.3
" "	Control without rabbit serum	3.27

0.2 ml of serial dilution of test serum were mixed with 0.2 ml of reagent at a constant concentration (final dilution of reagent = ⅙). All titers were calculated from the absolute dilution of test serum in the reaction mixture before addition of indicator cells.

all were inhibitory. In addition, data from assays using C'6-deficient rabbit serum as reagent did not yield a straight line when plotted according to the equation for a one step reaction (Fig. 3). This latter finding is consistent either with the presence of an inhibitor in C'6-deficient rabbit serum or with the possibility that more than one component is limiting.

These experiments with C'6-deficient rabbit serum showed that Balb/c female serum is relatively depleted in C'6. Consequently we thought that

Balb/c female serum might be used to assay C'6. The adequacy of this serum as a reagent for measuring C'6 depends on the assumptions that it supplies all components except C'6 in amounts which do not limit hemolysis and that the C'6 from the reagent serum does not contribute significantly to the C'6 titer. To test for this requirement, aliquots of serial dilutions from one pool of male Balb/c mouse serum were mixed with a constant concentration of reagent serum (Balb/c female). The titer of these mixtures was independent of the concentration of reagent serum employed (Table VI). These data were also

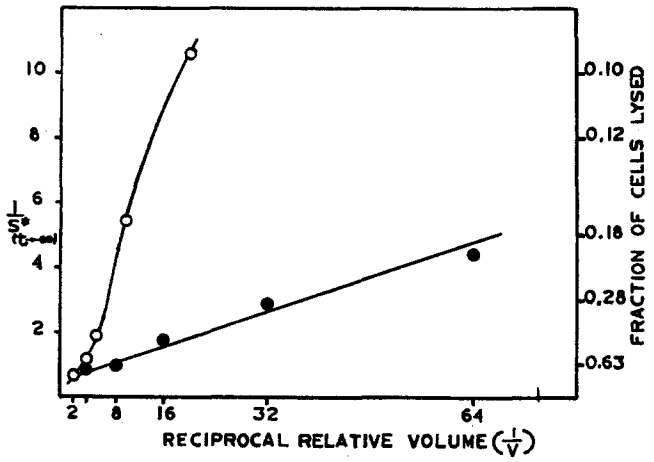

FIG. 3. Assay of C'6 in B10D2 "old line" mice using C'6-deficient rabbit serum or pooled sera from Balb/c female as reagent. Data are plotted according to the equation for a one step reaction. Open circle: mixtures contained C'6-deficient rabbit serum at a final dilution of 1/6 plus test serum. Closed circle: mixtures contain sera from Balb/c female mice at a final dilution of 1/8 plus test serum.

plotted according to the equation for a one step reaction. The data in Fig. 3 show that assay for C'6 with Balb/c female serum as reagent yielded a linear dose response curve. This finding is consistent with the conclusion that only one component is limiting in this assay.

Based on the evidence described in this section, we tentatively identified the component which is measured with Balb/c female serum as C'6. This identification is only tentative because more than two of the late-acting components may be affected by sex hormones.

The C'5 and C'6 Content of Sera from Normal and Castrated Mice of Both Sexes.—A mixture consisting of serum from Balb/c females and the non-hemolytic serum from genetically C'5-deficient male mice had about eight times as much whole C' activity as the serum of the Balb/c female alone (Table VII). Similar results were obtained in experiments in which C'^{m}-EDTA activity in-

stead of whole C′ activity was measured (Table VIII). These observations were interpreted to mean that at least one of the C′ components responsible for the male and female difference in complement titer is a late-acting component distinct from C′5.

We assayed the C′5 and C′6 content of sera from normal and castrated mice of both sexes. The data summarized in Table IX show that sera from male

TABLE VI

Assay of C′6 in Sera of Male Balb/c Mice using Different Concentrations of Balb/c Female Serum as Reagent

Test serum	Final dilution* of Balb/c female serum reagent	Titer (C′6H50)
Balb/c Male	1/12	21.0
" "	1/14	20.0
" "	1/16	20.6
" "	1/20	20.0

* Before addition of indicator cells. See Materials and Methods for experimental details.

TABLE VII

Whole C′^{m} Titrations of Serum Combinations

Test serum	Reagent	Titer
Balb/c M	Buffer (0.01 M EDTA)	43.3
" F	" 0.01 " "	3.3
B10D2 "old line" M	" 0.01 " "	0
" " " "	Balb/c F	25.6

Volumes of test serum were mixed with equal volume of reagent shown in column under Reagent, and the mixtures were serially diluted to assay complement activity. All titers are calculated from the absolute dilution of test serum in the reaction mixture before addition of indicator cells.

M: Normal male; and F: normal female.

Balb/c mice contain about three times more C′5 and about six times more C′6 than sera from female Balb/c mice contain. The C′5 and C′6 titers of sera from castrated male mice fell to levels usually found in sera from female mice. In sera from castrated female mice, the C′5 and C′6 titers rose to a level intermediate between the titers of a normal male and normal female. Comparison of the change of C′5 and C′6 titers after castration shows that C′6 titer is affected to a greater extent than the C′5 titer. Furthermore the change in C′6 titer after castration more closely parallels the changes observed in C′^{m}-EDTA activity after castration than does the change in C′5 titer (Table IX).

TABLE VIII

C'^{m}-EDTA Titrations of Serum Combinations

Test serum	Reagent	Titer
B10D2 "old line" M	Buffer (0.01 M EDTA)	0
Balb/c F	" 0.01 " "	2.0
" CM	" 0.01 " "	1.7
B10D2 "old line" M	Balb/c F	8.2
" " " "	" CM	7.6
Balb/c F	" "	3.9

Volumes of test serum were mixed with equal volumes of reagent shown in column under Reagent, and the mixtures were serially diluted to assay complement activity. All titers are calculated from the absolute dilution of test serum in the reaction mixture before addition of indicator cells.

M: normal male; CM: castrated male; and F: normal female.

TABLE IX

C'5, C'6, and C'^{m}-EDTA Titers in Sera from Normal and Castrated Balb/c Mice of Both Sexes

Mice	C'5	C'6	C'^{m}-EDTA
Male	54.8	26.2	20.2
Castrated male	20.8	4.87	3.37
Female	15.0	3.77	2.71
Castrated female	36.9	26.1	14.7

C'5 was assayed using sera from B10D2 "old line" mice as reagent (1/3). C'6 was assayed using sera from Balb/c females (1/8). All mice were castrated 6 months before these assays were performed.

TABLE X

Effect, In Vivo, of Exogenous Testosterone and Estradiol upon the C'5 Content of Mouse Sera

Mice	Treatment	Titer-C'5
B10D2 "new line" F	Testosterone	26.0
	Vehicle	8.4
B10D2 "new line" M	Testosterone	43.6
	Vehicle	32.6
B10D2 "new line" M	Estradiol	14.1
	Sesame Oil	21.8

B10D2 "old line" M (1/3) was the reagent used in all assays.

M: male; and F: female.

The Effect, In Vivo, of Exogenous Testosterone and Estradiol on C'5 and C'6 Activity.—Both male and female B10D2 mice of the "old" and "new" lines were given testosterone or estradiol. Control mice were given the appropriate vehicle. All animals received four subcutaneous injections equally spaced over a 2 wk period. The sera of the "old line" mice were assayed for C'6 and the sera from "new line" for C'5. The results of these experiments are summarized in

TABLE XI

Effect, In Vivo, of Exogenous Testosterone and Estradiol upon the C'6 Content of Mouse Sera

Mice	Treatment	Titer-C'6
B10D2 "old line" F	Testosterone	6.1
	Vehicle	1
B10D2 "old line" M	Testosterone	16.0
	Vehicle	12.8
B10D2 "old line" M	Estradiol	2.8
	Sesame Oil	9.8

Balb/c female serum, final dilution 1/8 was used as the reagent in all assays.
M: male; and F: female.

TABLE XII

The Effect of Deficiency of C'5 and C'6 on the Rejection of Male Balb/c Skin Graft by B10D2 Mice

Recipient animals	No. of animals	Day of rejection			
		9	10	11	Average
B10D2 "New line" male	10	2	5	3	10.1
" " " castrated male	10	1	5	4	10.3
" "Old line" male	11	1	2	8	10.6
" " " castrated male	11	1	4	6	10.5

Tables X and XI and show that the activity of both C'5 and C'6 is increased by administration of testosterone and depressed by administration of estradiol. The results are consistent with earlier observations on the effect of these hormones on whole C'^{m}-EDTA activity.

The Rejection of Homografts by Mice Deficient in C'5 and C'6.—Mice deficient in C'5 (B10D2 "Old line," DBA/2) have normal Arthus reaction (15, 16), normal homograft rejection (15, 16), normal phagocytic activity (17), and normal herd immunity (18). These mice, deficient in C'5, can be made relatively deficient in C'6 by castration. We have studied the course of homograft rejection in animals deficient in C'5, in animals deficient in C'5 and C'6, and

in normal animals. The skin grafts were taken from male Balb/c mice, a strain which is H2 compatible with the recipient mice. Approximately 1 cm^2 full thickness skin grafts were held in place with metal clips. For the first 4 days the grafts were protected with Band Aids. The day of complete escharification of the graft was recorded as the day of graft rejection. The results, summarized in Table XII, show that there was no significant difference in the time of rejection of skin in all four groups of mice.

DISCUSSION

We have reported that the administration of testosterone or estradiol to mice influences the titer of at least one of the serum complement components which act after the first, second, and fourth components (9). The overall titer of these late-acting components (C'^m-EDTA), usually 8–10 times greater in males than in females, increased 1.5–4 times after treatment of mice with testosterone. The C'^m-EDTA titer of the serum of castrated males or males treated with estradiol fell to levels normally found in intact female mice. We have developed assays for two of the late-acting components, and have shown that both of these components are affected by castration and by the administration of testosterone and estradiol. Identification of both these components required the use of serum deficient in one complement component. Serum from B10D2 "old line" mice were used to identify one component. B10D2 "old line" mice are thought to lack C'5 on the basis of gel diffusion studies. Until the possibility that sera from these mice lack other components as well has been excluded, identification of one of these two late-acting components as C'5 is uncertain. Identification of the other component as C'6 required the use of C'6-deficient rabbit serum and is subject to some uncertainty because of evidence which raised the possibility of a complement inhibitor in the C'6 rabbit serum. Whether the inhibitory effect of the C'6-deficient serum is due to the presence of an inhibitor or the result of mixing rabbit and mouse components is not known. In either case the inhibitory effect precludes use of this reagent to measure the component distinct from C'5 which is affected by sex hormones. Balb/C female serum may be used in place of C'6 rabbit serum and can be used to measure a component distinct from C'5. This component is tentatively identified as C'6. Late-acting components other than these two may be affected by sex hormones, but their identification awaits the development of suitable assays.

To obtain evidence that only one component was being measured in these assays, the data were evaluated by use of the equation for the one step reaction which, in turn, is based on the single site theory of immune hemolysis (14). Linear plots, indicating that only one component was limiting, were obtained from data of assays of C'5 and C'6 with mouse reagents. Using these assays we have found that the titer of C'5 in sera of male mice is two to four times

greater than the titer of this component in female mice. The titer of C'6 in sera of male mice is 6 to 12 times greater than the titer of this component in female mice.

The differences in C'5 content of sera of male and female mice are not sufficient to account for the observed difference in C'^{m}-EDTA titer of mouse sera since the C'^{m}-EDTA titers of the male sera may be 10 times higher than those of female sera. Based on these observations, we propose that the sex differences and the changes caused in C'^{m}-EDTA levels by hormone treatment or castration are mainly due to differences and changes in serum content of a component other than C'5, presumably C'6.

MuB1 is thought to be C'5 (7). Urbach and Cinader have measured the concentration of MuB1 in mouse sera (6) by a single diffusion ring test (19). These workers found that the concentration of MuB1 in normal males was about twice as high as that of normal females. The concentration of MuB1 in sera from castrated males approached the level found in normal females. Administration of testosterone to normal male and female mice and to castrated male and female mice increased the MuB1 concentration in the sera of these animals two- to threefold. Our findings, based on the hemolytic assay of C'5, are in agreement with their results.

These authors also found that estradiol caused a slight but significant increase in the MuB1 concentration in the sera of all mice except in the sera of sham-operated male mice. In contrast we observed that estrogen depressed the concentration of C'5. The cause of the discrepancy between Cinader's findings and ours is unknown but may be due to differences in mice, dose schedules, and assays. In addition, the possibility cannot be excluded that MuB1 and C'5 are two distinct molecules.

The administration of testosterone increased and the administration of estradiol decreased the C'6 titer of B10D2 "old line" mice which lack C'5 on a genetic basis. Sera from B10D2 mice, genetically deficient in C'5, did not acquire hemolytic activity after these mice were treated with large doses of testosterone. The C'6 titer in sera of C'5-deficient mice, however, increased in the expected manner after testosterone treatment.

Homograft rejection is the only biological process which we have studied in mice deficient in these two complement components. Despite the absolute deficiency of C'5 and the relative deficiency of C'6, the course of homograft rejection was unchanged in these mice.

SUMMARY

The titer of late-acting complement components in sera from male mice is 8–10 times higher than the titer of sera from female mice. Using assays developed to measure the serum content of two of the late-acting components, we have shown that this difference is due to the effect of androgen and estrogen

on these two late-acting complement components. These two components have been tentatively identified as C'5 and C'6. Androgen and estrogen have greater effect on C'6 than on C'5. The possibility has not been excluded that still other of the late-acting complement components are affected by androgens and estrogens.

The course of homograft rejection was unchanged in mice deficient in C'5 and C'6.

We thank Mr. M. O. Harris for able technical assistance.

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