The sex-dependent differences in serum levels of complement components have been reported concerning C4 (1), C5 (2, 3), C6 (3), C4-binding protein (C4-bp) (4), and sex-limited protein (Slp) (5) in the mouse complement system. The serum levels of these components in adult mice are always higher in males than in females within each inbred strain and are strongly under hormonal control. With regard to C5, young male and female mice have the same serum levels of C5, and the levels increase with age, most markedly in males and less in females (2). After sexual maturity, the ratio of C5 in serum of males vs. females is greater than 2:1. Injection of testosterone increases the levels of C5 in both males and females, whereas castration or injection of estradiol decreases the levels in males to those in females (3, 6). These reports, however, reveal only the quantitative difference but not the qualitative one.

During attempts to detect polymorphism of mouse C5 by isoelectric focusing (IEF), we found the difference in electrophoretic patterns of C5 between males and females. Females of all strains tested had one C5 band, but males had two C5 bands, one of which was identical to the female band. The expression of the other C5 band in males was shown to be influenced by sex hormones, indicating that this C5 is actually responsible for the sex-dependent difference.

Materials and Methods

Mice. BALB/c, C3H/He, and C57BL/6 mice were purchased from Shizuoka Animal Laboratory Center (Hamamatsu, Japan). A/He, AKR, A.SW, C57L, CBA, DBA/1, DDD, and SJL mice were supplied from the animal breeding unit of Institute of Medical Science, University of Tokyo (Tokyo). SWR mice were obtained from National Institute of Genetics (Mishima, Japan).

Plasma. 0.4 ml of blood of adult mice was collected by heart puncture into a syringe containing 0.1 ml of a solution consisting of 0.1 M ethylenediamine tetraacetic acid (EDTA)-Na2 and 0.1 M L-aminocaproic acid (EACA). Blood of neonatal mice was obtained by decapitation into a pasteur pipette coated with heparin and then transferred to a centrifuge tube containing EDTA and EACA. For periodic bleeding, blood was obtained from the retrobulbar plexus of the eye. After centrifugation, the plasma was stored in aliquots at -70°C.

Antisera. Alloantiserum to mouse C5 was obtained by immunizing A/He mice with serum from C57L mice incorporated in Freund’s complete adjuvant as described in (2).

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Abbreviations used in this paper: C, complement; C4-bp, C4-binding protein; EACA, L-aminocaproic acid; EDTA, ethylenediamine tetraacetic acid; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Slp, sex-limited protein.
To obtain rabbit antiserum to mouse C5, the alloantiserum was incubated with serum from BALB/c mice at equivalence. The resulting antigen-antibody precipitates were washed, emulsified in Freund's complete adjuvant, and injected into rabbits. The antiserum thus obtained was absorbed by passage through a column of Sepharose 4B coupled with C5-deficient mouse serum. Both alloantiserum and rabbit antiserum to C5 showed similar results in immunofixation and crossed immunoelectrophoresis, but the latter antiserum produced stronger precipitation. Therefore, the rabbit antiserum was used for the experiments in the present paper.

**IEF.** All plasma samples to be subjected to IEF were desialated by incubation at 37°C for 60 min with neuraminidase from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5 U/ml. IEF was performed in agarose gel (1.5 × 90 × 120 mm) consisting of 1% (wt/vol) agarose (Agarose IEF; Pharmacia Fine Chemicals AB., Uppsala), 0.2% (vol/vol) LKB Ampholine, pH 3.5–10, 1.75% (vol/vol) Ampholine, pH 5–8, 10% (wt/vol) sorbitol, and 1% (vol/vol) Nonidet P-40. 5 μl of desialated plasma samples were applied into wells of 2.5 mm in diameter 30 mm apart from the cathodic end. Sponge wicks moistened in a solution containing 2% Ampholine, pH 5.5–10, and 1% Nonidet P-40 were used as electrodes. Electrophoresis was carried out at 8°C for 90 min with a constant wattage of 4 W (upper voltage of 400 V). Under these conditions C5 was not yet focused at its isoelectric point, but electrophoretic patterns exhibited a discrete difference between male and female plasma. Plasma samples without desialation also showed this sexual dimorphism, but less discretely.

**Immunofixation with Anti-mouse C5 Antiserum.** After IEF, the agarose gel plate was overlaid with Whatman No. 3 filter paper moistened with anti-mouse C5 antiserum. The plate was incubated in a humid chamber at 37°C for 60 min, left at 4°C overnight, pressed with absorbent paper, washed in isotonic saline, dried, and then stained in 0.25% Coomassie Brilliant Blue.

**Crossed Immunoelectrophoresis.** After IEF, the agarose gel was cut into strips of 10 mm in width and 50 mm in length, and the gel strips were placed on a second agarose gel plate (1.5 × 50 × 50 mm), which consisted of 0.8% agarose containing anti-mouse C5 antiserum in 25 mM Veronal buffer with 2 mM EDTA, pH 8.6. After electrophoresis at 3 V/cm and 15°C for 18–20 h, the gel was pressed, dried, and stained.

**Analysis of Molecular Weight of C5.** The desialated EDTA-plasma of male or female BALB/c mice was fractionated by gel-filtration on a Sephadex G-200 column. The fractions containing C5 were pooled, radiolabeled with 125I by the method of Chloramine-T (7), and immunoprecipitated with rabbit anti-mouse C5 antiserum or with normal rabbit serum as a control. The resulting immune precipitates were adsorbed onto *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) (8). After washing, the radiolabeled C5 molecules remained bound to the bacteria were eluted as described (9), and analyzed by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) followed by radioautography.

**Quantitation of C5.** The levels of C5 protein in mouse plasma were determined by rocket immunoelectrophoresis against antiserum to mouse C5.

Hemolytic activity of C5 in plasma samples was determined using EAC114 (11), "xYC2h" (12), and a reagent containing guinea pig C3, C6, C7, C8, and C9 (13). Titers were expressed as the reciprocal of dilution of the sample giving 50% lysis. To measure hemolytic activity of samples separated by IEF, the agarose gel was cut into strips of 10 mm in width and then the strips were cut into slices of 1.5 mm in length. The slices were immersed in 1 ml of isotonic saline followed by incubation at 4°C for 20 h. The eluates were diluted to 1/40 and then assayed for hemolytic activity of C5. The activity was shown using the degree of cells lysed.

**Injection of Hormone.** Testosterone and estradiol were purchased from Teikoku-zoki Co., Tokyo. Sesame oil used as a carrier of these hormones was kindly supplied by the same company. Testosterone and estradiol were weekly injected subcutaneously in the back in the dose of 2.5 mg and 0.1 mg, respectively, per mouse for 7 wk. Control mice were given sesame oil.
Results

**Sex-associated Difference in Electrophoretic Patterns of C5 in Mouse Plasma.** Desialated plasma samples of males and females of BALB/c, C3H/He, C57BL/6, CBA, DBA/1, and SJL mice were subjected to IEF, followed by immunofixation with anti-C5 antiserum. Four to seven mice of each sex of each strain at 8–10 wk of age were individually examined. As shown in Fig. 1, two bands, an acidic and a basic, were observed in males, while only one was detected in females. The female band and the male basic band are likely to have the same electrophoretic mobility. No band was found in C5-deficient mice, such as A/He, AKR, A.SW, DDD, and SWR.

**Identification of Male Basic C5 Band with Female C5 Band.** The following samples taken from BALB/c mice were subjected to IEF; male plasma, female plasma, and a mixture of both (1:1). Each sample was run in triplicate. After separation the triplicate samples were subjected to: (a) crossed immunoelectrophoresis against anti-C5, (b) immunofixation with anti-C5, and (c) assay of C5 hemolytic activity.

On crossed immunoelectrophoresis, male plasma produced a single precipitin line with bi-peak (Fig. 2A), while female plasma showed a single line with one peak (Fig. 2B). The mixture of male and female plasma also exhibited a single precipitin line with bi-peak, but the acidic peak was reduced to half (Fig. 2C). In addition, the hemolytic activity of C5 corresponded well to the precipitation patterns.

Next, male and female plasma samples were fractionated in Sephadex G-200 columns. C5 protein in both plasma appeared in the ascending limb of the second
Fig. 2. Crossed immunoelectrophoresis (IEP) and hemolytic activity patterns of C5 in the plasma from 8-wk-old BALB/c male and female mice. Desialated samples of male plasma, female plasma, and a mixture of both (1:1) were subjected to IEF in triplicate. After IEF, the first gel strip of the triplicate, 10 mm in width, was transferred to a second agarose gel containing 0.8% anti-C5 antiserum and then subjected to crossed immunoelectrophoresis. The second gel strip was immunofixed with anti-C5. The third gel strip was cut into slices of 1.5 mm in length and immersed in isotonic saline. The eluates were assayed for hemolytic activity of C5. A, male plasma; B, female plasma; C, a mixture of male and female plasma.

protein peak with apparent mol wt of 200,000. The fractions containing C5 were radiolabeled, immunoprecipitated, and analyzed by SDS-PAGE. Fig. 3 shows the results. Male C5 was dissociated into two bands as female C5, and no difference was detected in the molecular weights between male and female C5.

From these results we draw the following conclusions: (a) male basic C5 is identical with female C5, and (b) acidic C5 is characteristic of male mice in spite of the indistinguishability from basic C5 of antigenicity, functional activity, and molecular weight.

C5 of Neonatal Mice of Both Sexes. Since the levels of C5 in serum have been shown not to be different in young mice of both sexes (2), we compared the precipitation patterns of C5 between neonatal male and female mice. Plasma samples from 2-d-old BALB/c mice of both sexes were analyzed by crossed immunoelectrophoresis against anti-C5. As shown in Fig. 4, basic C5 was observed in both males and females at the same levels, whereas acidic C5 was hardly detected. No significant difference was observed in the functional activity and the protein concentration of C5 between these male and female mice (Table I). These results suggest that the acidic C5 is under the control of sex hormones. This was examined in the next experiments.

Effect of Exogenous Testosterone and Estradiol on the C5 Precipitation Patterns. Testosterone or estradiol was injected weekly into both male and female BALB/c mice. Plasma samples taken at intervals from individual animals were analyzed by crossed immunoelectrophoresis against anti-C5, and assayed for the
FIGURE 3. Radioautogram of 7.5% SDS-PAGE of partially purified mouse male and female C5. Plasma samples from BALB/c were fractionated in Sephadex G-200 columns. The ascending limb of the second peak was radiolabeled and immunoprecipitated with normal rabbit serum (tracks A and C) or rabbit anti-mouse C5 (tracks B and D). Both male (track B) and female (tracks D) C5 exhibited the α- and β-chains under reducing conditions.

FIGURE 4. Crossed immunoelectrophoresis patterns of C5 in the plasma from 2-d-old BALB/c male and female mice. Desialated plasma samples were subjected to IEF, followed by crossed immunoelectrophoresis on agarose gel containing 0.4% anti-C5 antiserum. As controls, plasma samples from 8-wk-old BALB/c male and female mice were examined.

hemolytic activity of C5. The results are shown in Fig. 5 and 6. 1 wk after the first injection of testosterone, acidic C5 newly appeared in females and increased in males. By 7 wk the female acidic C5 increased to the same levels as those of male acidic C5, and the precipitation pattern of female C5 became similar to that of male C5. When injecting estradiol, male acidic C5 decreased 1 wk after
TABLE I

Hemolytic Activity and Concentration of C5 in Plasma from Neonatal Male and Female Mice*

| Age | Sex  | Hemolytic activity of C5 | Concentration of C5 protein* |
|-----|------|--------------------------|-----------------------------|
|     |      | U/ml         | %                           |
| 2 d | Male | 850          | 16.6                        |
|     | Female | 750          | 14.7                        |
| 8 wk | Male | 5,250        | 100                         |
|     | Female | 1,980        | 39.4                        |

* The samples tested were the same plasma from mice used in Fig. 4, but not desialated.

The relative concentrations of C5 protein were determined by measuring the precipitation area obtained by rocket immunoelectrophoresis, and expressed as a percentage of the plasma from 8-wk-old male mice.

the first injection, and disappeared by 7 wk. On the other hand, basic C5 decreased with the injection of testosterone, but was not influenced by estradiol. Injection of testosterone resulted in an increase of the levels of hemolytic activity of C5 in male and female plasma. In contrast, estradiol diminished the functional levels of male C5, but showed no effect on those of female C5. In parallel with the change in hemolytic activity was a corresponding change in C5 protein concentration (results not shown).

Thus, it is clear that the expression of acidic C5 is under the control of testosterone.

Discussion

This paper describes sexual dimorphism of murine C5. The observed dimorphism could be due to differences in the amino acid sequence or to postsynthetic modifications. We definitively exclude the possibility that the C5 dimorphism is artefactual heterogeneity by some technical irregularities. First, the acidic band is not an unidentified male plasma protein reacting with a contaminating antibody, since crossed immunoelectrophoresis using anti-mouse C5 revealed complete antigenic identity of both acidic and basic C5 (Fig. 2). Second, the dimorphism described here is not an artefact by neuraminidase treatment, because untreated male plasma showed two C5 bands, but less discretely. Third, it is unlikely that C5b, generated during the experimental procedures, formed complexes with the subsequent components, because the hemolytic activities of both acidic and basic C5 corresponded well to the concentration of C5 protein (Fig. 2).

Our observations clarify the nature of the sex-dependent difference in the serum concentration of C5. Cinader and his colleagues (2, 14) have reported that there is no difference in the serum concentration of C5 between young male and female mice, but thereafter the levels of C5 increase gradually in males to reach approximately twice the levels of that in females. Further, they have found that administration of testosterone increases the concentration of C5 in mice of both sexes (6). Similar observations have been reported concerning the hemolytic activity of C5 in mice (3). Our findings in this paper, based on electrophoretic
FIGURE 5. Changes in crossed immunoelectrophoresis patterns of C5 in the plasma from male and female mice injected with sex hormones. 8-wk-old BALB/c mice received the first injection of sex hormones, followed by weekly injections for 7 wk. Control mice were given sesame oil as a carrier. Desialated samples were subjected to IEF, followed by crossed immunoelectrophoresis on agarose gel containing 0.7% anti-C5 antiserum. The patterns of male mice injected with testosterone are shown in A, female with testosterone in B, male with estradiol in C, and female with estradiol in D. Patterns of control mice are not shown, since no changes were observed.

patterns of C5, are not only in agreement with their results, but also visibly account for their observations. That is to say, the development of the acidic C5 in mouse serum reflects the steady increase of C5 in males with sexual maturity, and the consequent sex-dependent difference in the serum levels of C5.

Further, the similar idea might be applicable to the sex-dependent differences in the serum levels of several components of mouse complement such as C4 (1), C6 (3), and C4-bp (4). The predominant presence of male hormone, by sexual maturity in males or by administration of testosterone, increases the serum levels of these components. The inferior presence of male hormone, by castration of males and/or by administration of estradiol, decreases the levels in males to the same as those in females. Thus, in analogy with C5, sexual dimorphism might be present in C4, C6, and C4-bp.

How does testosterone produce the sexual dimorphism of mouse C5? We must consider first a possibility of posttranslational modification in which a testoster-
FIGURE 6. Changes in hemolytic activity of C5 in the plasma from male and female mice injected with sex hormones. Plasma samples in Fig. 5 were assayed without desialation for hemolytic activity of C5. Each curve represents the results of the hemolytic titration of C5 in the plasma from one mouse in each group: male injected with testosterone (○), male with estradiol (●), males with sesame oil (▲), female with testosterone (○), female with estradiol (□), and female with sesame oil (△). Arrows indicate hormone injections.

one-dependent factor converts basic C5 to acidic C5, since in mice given testosterone the increase in serum levels of acidic C5 is accompanied by the decrease in basic C5 (Fig. 5). The findings by Ooi and Colten (15) may support this possibility. They have demonstrated that peritoneal macrophages from male mice generate only one form of intracellular pro-C5, but secrete two forms of extracellular pro-C5. One has the mobility corresponding to that of the intracellular pro-C5 and the other migrates in a more anodal position. Alternatively, we may postulate two genetic loci for mouse C5; one locus encodes basic C5 and the other, turned on by testosterone, encodes acidic C5. The exact mechanism involved in the hormonal regulation of C5 production remains to be determined.

Summary

Sexual dimorphism of mouse complement component 5 (C5) was detected by isoelectric focusing of desialated ethylenediamine tetraacetic acid (EDTA)-plasma on agarose gel, followed by immunofixation with anti-mouse C5. Male plasma displayed two C5 bands, basic and acidic, while female plasma had only a basic C5 band. In all mouse strains tested except C5-deficient strains, the identical patterns of the dimorphism were obtained. The basic and acidic C5 were antigenically and hemolytically indistinguishable from each other. In the neonatal mice of both sexes, the basic C5 was observed at the same levels, but the acidic C5 was only slightly detected. Injection of testosterone into mice resulted in an appearance of the acidic C5 in females and its increase in males. By contrast,
injection of estradiol decreased the acidic C5. These results indicate that the expression of the acidic C5 is under the control of testosterone.

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