Hormonal Control of MuBl Concentration.* (31250)

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MuBl is a serum antigen of the mouse(1) which forms part of the complement system (2,3,4) and is probably identical with C'5 (5). In the serum of adult animals its concentration is always greater in the male than in the female(2,6, see also 7). This sex-dependent difference in antigen concentration cannot be due to X chromosome linkage, since this would result either in a larger concentration of MuBl in females or in an identical concentration of MuBl in animals of both sexes(8,9,10). Furthermore, back-cross experiments do not reveal a relation between the presence or absence of MuBl and the sex of parents or the sex of offspring(2,6). It seems likely that the amount of MuBl is under hormonal control. Support for this view is provided by the fact that the levels of the antigen are *not* different in very *young* animals of different sexes(11). To examine this question we have subjected animals to various hormonal stimuli, and measured the changes in concentration of MuBl. The results provide support for the thesis that the levels of this serum protein are determined to some extent by the hormonal state of the animal.

Materials and methods. Testosterone propionate was obtained from Charles E. Frosst & Co., Montreal, Quebec. Medroxy progesterone acetate was supplied by the Upjohn Co. of Canada, Don Mills, Ontario. Oestradiol monobenzoate was supplied by British Drug Houses (Canada) Ltd., Toronto, Ontario. Sodium pentobarbital was obtained from Abbott Laboratories Ltd., Montreal, Quebec.

Glycine buffer (pH8.4) consisted of 14.260 g glycine, 11.115 g NaCl and 100 ml 0.1 M NaOH, made up to 2 litres with glass distilled water.

Glycine agarose (1.1% w/v agarose) was prepared by adding to 1 litre of glass distilled water, 2 g sodium azide and 24 g agarose. This mixture was kept at 100°C for 45 minutes, and was mixed at 56°C with 1 litre of filtered glycine buffer.

MuBl antisera were obtained by immunizing female mice of strain AHe/J with C57L/J ♀ sera, incorporated in complete Freund's adjuvant. The mice were bled from the tails; a pool was prepared from bleedings taken over 237 days after 8-17 injections.

Male and female DBA/1J mice, 6 weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Mice were anaesthetized by i.p. injections with pentobarbital (0.6 mg per 10 g body weight). Gonadectomy was performed through appropriate flank incisions on the right and left side of the animal. After the peritoneum was incised, the gonads were brought to the opening and removed. The skin incisions were then closed with metal clips. Sham surgery consisted in bilateral flank incisions. The peritoneal cavity was entered and the surgical incision in the skin was then closed with metal clips. Some animals received no further treatment. Others were injected intramuscularly with hormones after the operation and at weekly intervals thereafter. The relative doses of different hormones were chosen by comparison with effective doses in man; detailed dose-response curves were only determined with testosterone propionate. The weekly dose of hormones was either 10 mg testosterone propionate, 0.25 mg oestradiol

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monobenzoate or 5 mg medroxy progesterone. Each of the above treatments was carried out with groups of 10 animals. The animals were bled from the tail at intervals of 4 weeks. Blood was collected in test tubes immersed in an ice-water bath, kept at 0°C for 1 hour, and then centrifuged at +2°C and 2,000 rpm. The sera were stored at −10°C.

The concentration of MuBl was measured by a single diffusion-ring test, based on the method of Fahey *et al*(12). MuBl antisera (0.38 ml) and glycine buffer (1.12 ml) were mixed at 56°C and added to glycine agarose (1.5 ml). The carefully mixed fluid was poured into the agar diffusion plate and allowed to set. For this purpose "Immuno-Plates," supplied by Hyland Laboratories, Los Angeles, Calif., were used. A series of 24 evenly spaced wells (diameter: 3.7 mm) were punched into the gel and were filled with antigen to the rim. To establish suitable conditions for the test, various dilutions of a standard serum were incubated for varying periods at 20.8 ± 0.2°C; the shortest incubation time which gave reproducible results was finally selected. The conditions chosen for the assay were as follows: The agar diffusion plates were sealed, kept for 4 hours at 20.8 ± 0.2°C and were then photographed in the Cordis immuno-diffusion camera. Photographs were projected, the ring-widths of the opaque antigen-antibody rings were determined and corrected for enlargement. The resulting measurements were evaluated by comparison with a calibration curve in which ring widths were plotted against the logarithm of various concentrations of a standard preparation. The ring width obtained with a pool of sera from 6-month-old male DBA/1J animals was taken as the 100% value.

Results. The MuBl concentration of male DBA/1J mice, which had been sham operated, increased by 50% within 4 weeks of the operation; that of female animals showed a slight decrease (7%) and the relative concentration in males became about twice that of females. If male mice were gonadectomized, the increase shown by the sham operated animals did not occur and there was even a slight decrease in concentration (14%) which became essentially the same as that of females. Having thus found that the concentration differences between male and female animals depended on the functioning gonad of the male, we inquired next whether the normal slow increase with age of the MuBl content of a male's serum could be accelerated by testosterone. We determined MuBl concentration in animals treated with varying quantities of testosterone propionate. Groups of 6-week-old males were left untreated or given a single injection. Within 11 days of the injection with 1-10 mg a marked increase of MuBl concentration could be noted (Table I). Thus the increase of MuBl with age might be attributable to androgen-mediated stimulation.

TABLE I. Increase in MuBl After a Single Injection with Testosterone Propionate. Male 6-wk-old DBA/1J mice were employed, and 5 animals formed one treatment and bleeding group; italicized values are significantly different from those of untreated controls (p is the probability of the level of significance, *i.e.* of the chance of committing a type I error in rejecting the hypothesis).

	mg testosterone propionate			
Days after injection	0	0.1	1.0	10
	MuBl concentration in % of standard			
7	38	53 .05<p<.10	*62* p<.01	*68* p<.01
11	36	40 .20<p<.25	*48* .025<p<.05	*78* p<.01
16	40	39 .60<p<.70	55 .05<p<.10	*80* p<.01

In the following experiments, groups of 10 animals of both sexes were sham operated or gonadectomized and were left untreated or received, after the operation, weekly injections with one of 3 steroid hormones (Fig. 1, 2).

Treatment with testosterone propionate induced a dramatic increase in the MuBl concentration of sham operated or gonadectomized females. This increase had reached completion after 4 weeks of treatment and thereafter a fairly constant level was maintained. Male animals reacted with a similar increase and the MuBl concentration of testosterone-treated gonadectomized males, after 4 weeks of treatment, was essentially the same as that of testosterone-treated sham operated animals. In males which had undergone sham

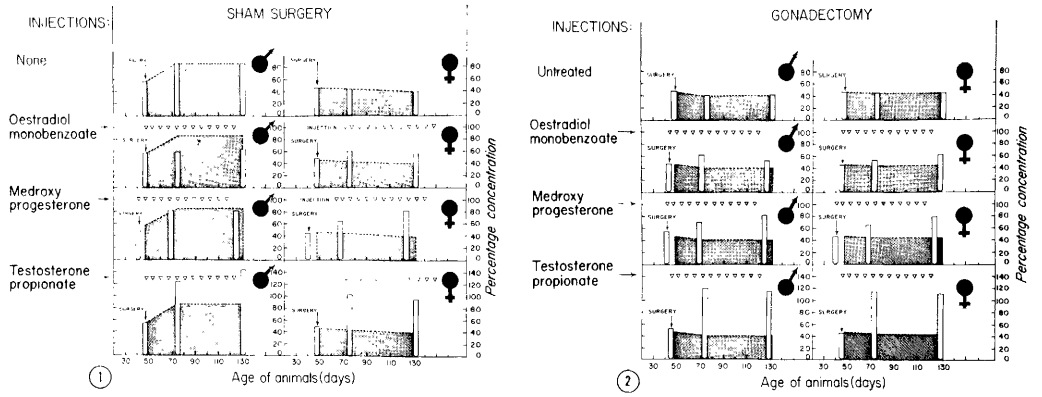

FIG. 1. Effect of various hormone preparations on serum concentration of MuBl in sham operated animals. Open triangles mark time of injections. MuBl concentrations are expressed as percentage of a standard preparation. For each treatment, indicated on left-hand side of Figure, concentration of MuBl is shown by open bars. The first bar in each box indicates concentration before sham operation and before injections, the next 2 bars indicate concentration after sham operation and treatment with hormones. Stippled areas indicate MuBl concentration in serum of animals which have not been injected with hormones. Contours of stippled areas are based on measurements of MuBl in sera taken before sham operation and 4 and 12 weeks after operation.

FIG. 2. Effect of various hormone preparations on serum concentration of MuBl in gonadectomized animals. Open triangles mark times of injection. MuBl concentrations are expressed as percentage of a standard preparation. For each treatment, indicated on left-hand side of Figure, concentration of MuBl is shown by open bars. The first bar in each box indicates concentration before gonadectomy, and before any injections, the next 2 bars indicate concentration after gonadectomy and treatment with hormones. Stippled areas indicate MuBl concentration in animals which have not been injected with hormones. Contours of stippled areas are based on measurements of MuBl in sera taken before gonadectomy and 4 and 12 weeks after operation.

surgery an apparent further increase was observed at the end of the twelfth week of treatment, but was not significantly different from the levels found in the earlier bleedings. Testosterone treatment did not bring about a significant change in the total serum protein nitrogen concentration of any of the above group of animals.

Medroxy progesterone acetate induced a relatively small, but significant concentration increase in sham operated females and in gonadectomized males and females as compared to the levels found in corresponding uninjected groups; it had no effect on the MuBl concentration of sham operated male mice.

Oestradiol monobenzoate induced an even smaller but still significant increase, in the MuBl levels of sham operated females and of gonadectomized females and males. However, in sham operated males, the MuBl concentration was significantly lower than in the corresponding uninjected male animals, and was identical with that of the other 3 oestrogen-treated groups of animals (Fig. 1, 2).

Discussion. The difference in MuBl levels of male and female animals indicates that the central genetic control of the concentration of this complement component is modified by a peripheral regulatory mechanism. In previous experiments a sex dependence has been found, not only in MuBl concentration but also in the number of hemolytic complement units(2,7). However, the ratios determined by these two measurements did not agree(2). Since the hemolytic effect is the result of the interaction of at least 9 factors(13), it is clear that fluctuations of MuBl, as one of these factors, will not necessarily be reflected in a proportionate change in the final reaction product. We have, therefore, relied here on the direct determination of MuBl concentration by its interaction with an isologous antibody.

The sex difference in MuBl concentration can be abolished by gonadectomy of the male or by administration of testosterone to the female. Indeed, testosterone led to an increase of MuBl concentration of all groups of animals, well beyond the highest values found in normal males. It is, therefore, apparent that

the serum levels of MuBl are under hormonal control. Most of our results may be explained by a direct effect of testosterone on the serum concentration of MuBl. The augmentation of MuBl concentration by medroxy progesterone might be attributed to the *in vivo* conversion of this component to 6-α-methyl testosterone. The reduction by oestrogen of the MuBl levels of sham operated males may be mediated through an indirect effect of oestrogen on the synthesis or action of testosterone. Oestrogen may suppress pituitary hormone production(14) and this, in turn, may result in decreased testosterone secretion by the testicles. Thus a dominant role of testosterone could account for most of our observations. However, one divergent finding indicates that a more complex interaction of hormonal factors may be involved. The small but significant oestrogen-induced increase in MuBl levels of gonadectomized females cannot be explained in terms of testosterone mediated action. Further experimental analysis may reveal the pathway of this effect, and refine the present conclusion that the concentration of MuBl is under a hormonal control in which androgens play an important part.

Sex differences in MuBl concentration gain added interest from the fact that hemolytic complement action may thus be modified through a fluctuation of one participant in a cascade of interlocking reactions. Indeed, the relatively low level of MuBl in the female may be responsible for low hemolytic complement levels(2) and may thus have a selective advantage in the evolution of the species. It is well known that antibodies cause little cell damage unless hemolytic complement intervenes(15). Thus the intriguing phenomenon of the survival of the foetal heterograft may partly depend on the relative ineffectiveness of the female complement system.

Summary. MuBl, a complement component of the mouse, occurs in higher concentration in male than in female animals. In the gonadectomized male, the level of MuBl is identical with that of the female, but can be restored to its original value and even increased above its normal concentration by administration of testosterone propionate. MuBl concentration of the female can also be increased by testosterone. Administration of medroxy progesterone acetate leads to a less pronounced increase in concentration. This is found in sham operated females, gonadectomized males and females, but not in sham operated males. Oestradiol monobenzoate slightly augments MuBl levels of gonadectomized female and male animals and of sham operated females. It stabilizes the MuBl concentration of sham operated males, thus causing a relatively lower concentration of MuBl in sham operated, oestrogen-treated males, than in sham operated uninjected males of identical age.

1. Cinader, B., Dubiski, S., Nature, 1963, v200, 781.

2. Cinader, B., Dubiski, S., Wardlaw, A. C., J. Exp. Med., 1964, v120, 897.

3. Rosenberg, L. T., Tachibana, D. K., J. Immunol., 1962, v89, 861.

4. Erickson, R. P., Tachibana, D. K., Herzenberg, L. A., Rosenberg, L. T., ibid., 1964, v92, 611.

5. Nilsson, U., Müller-Eberhard, H. J., Fed. Proc., 1965, v24, Part I, 620.

6. Cinader, B., Dubiski, S., Wardlaw, A. C., Genet. Res., 1966, v7, 32.

7. Terry, W. D., Borsos, T., Rapp, H. J., J. Immunol., 1964, v92, 576.

8. Lyon, M. F., Nature, 1961, v190, 372.

9. ———, Am. J. Hum. Gen., 1962, v14, 135.

10. Harris, H., 2nd Int. Conf. on Congenital Malformations, International Medical Congress, New York, 1964, 135.

11. Dubiski, S., Cinader, B., Proc. Soc. Exp. Biol. and Med., 1966, v122, 775.

12. Fahey, J. L., McKelvey, E. M., J. Immunol., 1965, v94, 84.

13. Cinader, B., Lepow, I., Antibodies to Biologically Active Molecules, Pergamon Press, Oxford, 1966.

14. Baggett, B., Engel, L. L., Savard, K., Dorfman, R. I., J. Biol. Chem., 1956, v221, 931.

15. Cinader, B., in Canad. Cancer Conf., Vol. 5, Academic Press, New York, 1963, 279.

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