95. *Immunological Studies of Pregnant Mare Serum Gonadotropin (PMSG). II*

Preparation of Pure Serum Gamma-globulin on Anti-PMSG Rabbit Serum

By Shigenori IKEMOTO,^{*)} Harutaka MUKOYAMA,^{**)}
Shozo SUZUKI,^{***)} and Tanemoto FURUHATA, M.J.A.^{****)}

(Comm. May 19, 1965)

A previous report¹⁾ described the injection of pregnant mare serum gonadotropin (PMSG) with Freund's adjuvant into a rabbit, inducing the production of an anti-PMSG rabbit serum which showed precipitin and agglutinin properties. By means of this precipitin test, the anti-PMSG rabbit serum may be used for immunological pregnancy test. The present paper reports on the immunoelectrophoretic analysis and immunochemical analysis of the anti-PMSG rabbit serum.

Materials and Methods. Anti-PMSG rabbit serum: The preparation of anti-PMSG rabbit serum, the method for immunization, the immunological reaction and the immunological pregnancy test, have been given in the first report.¹⁾ The storage condition was with 0.1% (w/v) sodium azide at 5°C.

Ultracentrifugation analysis: Anti-PMSG rabbit serum was clarified by centrifuge for 5 minutes at 3,000 r.p.m., and then centrifugalized for 10 hours at 144,700 g., 40,000 r.p.m. in a Hitachi RP-40-103 rotor with refrigeration.

The sedimentation was calculated by the method described by Campbell.²⁾

Immunoelectrophoretic analysis: Immunoelectrophoresis was carried out according to the method of Hirschfeld³⁾ under the following conditions; agar gel at 1%, veronal buffer at pH 8.6 and ionic strength 0.1, the potential gradient was 6 v/cm for 3 hours, dyed with amido black 10 B. The immunoelectrophoretic analysis was made using the L.K.B. apparatus.

Separation of serum fraction (DEAE batch method): The fraction was carried out according to the method of Stanworth⁴⁾ under the following conditions. 2.5 ml of anti-PMSG rabbit serum (centrifugation analysis fraction 3), previously dialysed for 16 hours at 4°C

*) Laboratory of Legal Medicine, National Research Institute of Police Science, Tokyo.

**) Laboratory of Animal Breeding, Tokyo University of Agriculture.

***) Professor, Tokyo University of Agriculture.

****) Director, National Research Institute of Police Science, Tokyo.

against one liter sodium phosphate buffer (pH 7.5, 0.01 M) containing 0.015 M sodium chloride, was added dropwise with continuous stirring to moist diethyl amino ethyl-cellulose (17 gm wet weight) which had been equilibrated and mixed with 2.5 ml of a similar buffer pre-cooled to 4°C. After the mixture had stood at 4°C for 5 hours, a further 5 ml of buffer was added with stirring and the resultant slurry was centrifugalized in a cooled tube at 3,000 r.p.m. for 15 minutes (fraction 1). A slightly less pure preparation (fraction 2) was obtained by a repeat rinse of the exchanger with a further 5 ml of phosphate buffer at pH 7.5.

Effect of 2-mercaptoethanol: The various test sera and their fraction (1 and 2) were treated with 2-mercaptoethanol (0.1 M) in phosphate saline buffer pH 7.0, by the method of Chan and Deutsch.⁵⁾ The mercaptan was removed by dialysis against phosphate saline buffer containing 0.02 M iodoacetamide, and the treated antiserum was examined for serological activity.

Results and Discussion. Ultracentrifugation analysis: Table I shows the results of ultracentrifugation analysis. The protein concentrations, F-1, F-2, F-3, F-4, and F-5 fraction were 1.4, 2.0, 5.0, 14.0, and 24.3 percent respectively. The precipitin titer of F-3 fraction was 50 IU, but no precipitin reaction was observed in F-1, F-2, F-4, and F-5. Results show that the anti-PMSG rabbit serum has a smaller molecular weight than other anti-A, B and anti-D saline agglutinin.²⁾

Immunoelectrophoretic analysis: Fig. 1 shows the immunoelectrophoretic patterns in agar gel given by the isolated antibodies.

Table I. Antibody titer and protein concentration of serum fraction obtained by centrifugation

Protein concentration						PMSG (IU/ml)
(%)						50
30						100
25						1,000
15						2,000
10						3,000
5						
Fraction	F-1	F-2	F-3	F-4	F-5	Uncentrifugation antiserum

Protein concentration: Hitachi hand protein refractometer.

The antigen antibody precipitin line started developing within 24 hours. Fig. 1, (a) and (b) indicate how the serum of pregnant mare, non-pregnant mare and gelding reacted with both the unabsorbed and absorbed anti-PMSG serum. Each antigenic serum gives two or three precipitin lines for unabsorbed antiserum. The serum of pregnant mare gave one precipitin line for absorbed anti-serum, but non-pregnant mare serum and gelding serum showed no reaction. Fig. 1, (c) indicates that the absorbed anti-PMSG rabbit serum reacted with the PMSG 1,000 IU per ml giving only one precipitin line. The immunoelectrophoretic pattern shows that the major constituent of the isolated antibody is γ -globulin.

Separation of serum fraction: Immunoelectrophoretic analysis was made according to the method previously described. Fig. 2 shows the separated serum fraction 1 (DEAE batch method) reacting with the PMSG hormone 1,000 IU per ml. The precipitin reaction of fraction 1 was a clear line. However the separated fraction 2 showed no reaction.

Fig. 1. Immunoelectrophoretic analysis with unabsorbed and absorbed anti-PMSG serum to the sera of pregnant mare, non-pregnant mare and gelding.

Fig. 2. Immunoelectrophoretic analysis with antiserum (DEAE batch method, fraction 1) and 2) to the PMSG.

Stanworth⁴⁾ described that fraction 1 and fraction 2 were composed entirely of γ -globulin (IgG(7S)-type). Small amounts of a β -globulin contaminant, probably siderophilin, can be detected by testing with a more potent specific antiserum against α and β -globulins. Quantitative gel-diffusion precipitin analysis with specific rabbits antisera against α_2 -glycoprotein and IgM(19S) type γ -globulin demonstrated that the γ -globulin fraction 1 and 2 were completely free of these high-molecular weight proteins. Therefore, this anti-PMSG rabbit serum was probably composed of IgG(7S) type γ -globulin.

Effect of 2-mercaptoethanol: Fig. 3 and Table II show that anti-PMSG rabbit serum revealing precipitin activity can be demonstrated to possess it even after being treated with mercaptan. Predominating average results presented in this table indicated that saline agglutinins were usually destroyed by such treatment and that the anti-PMSG rabbit serum and IgG(7S) agglutinin were not affected.

Chan and Deutsch⁵⁾ described that the IgG(7S) and IgM(19S) type γ -globulin of Rh antiserum possessing both saline and Coombs antibodies were separated by centrifugation in density gradient. The saline agglutinins associated with the IgM(19S) type γ -globulin

1: PMSG 1,000 IU (IU/ml),   2: PMSG 500 IU (IU/ml),
3: PMSG 100 IU (IU/ml),   4: buffered saline (pH 7.5),
S: anti-PMSG serum (DEAE batch method fraction 1).

Fig. 3. Effect of 2-mercaptoethanol treated anti-PMSG serum.

Table II. Effect of 2-mercaptoethanol on anti-PMSG serum and control sera

Material sera		Test method	Titer	
			Untreatment	Treatment
Anti-PMSG		P	50 (IU)	50 (IU)
		G	100 (IU)	100 (IU)
		A	500 (IU)*	500 (IU)*
Saline agglutinin	Anti-A (IgM)	A	128	0
	Anti-B (IgM)	A	256	0
Coombs agglutinin	Anti-D (IgG)	A	32	32
	Anti-E (IgG)	A	16	16

P: precipitin reaction, G: agar gel diffusion, A: hemagglutination.

*: Hemagglutination with at a corted tannic acid treatment of formalinized sheep red cells.

Anti-A, B sera: positive on anti-IgM serum.

Anti-D, E sera: positive on anti-IgG serum.

were destroyed by 2-mercaptoethanol. After treatment, no residual antibody including Coombs reactivity was found. In this study, the anti-PMSG rabbit serum association with the fraction 1 (DEAE batch method) was not destroyed by 2-mercaptoethanol. Accordingly, this anti-PMSG rabbit serum is probably IgG(7S) type γ -globulin.

Summary. Gamma-globulin was separated from the anti-PMSG rabbit serum by using diethylaminoethyl-cellulose anion exchanger (DEAE batch method). The studies of immunoelectrophoretic analysis and treatment of 2-mercaptoethanol show that the major constituent of the isolated antibody was IgG(7S) type γ -globulin.

Acknowledgement: The authours wish to thank Dr. M. Watanabe, Dr. M. Kitahama, and Dr. S. Ohkuma of the National Research Institute of Police Science.

References

- 1) Ikemoto, S., Mukoyama, H., Suzuki, S., and Furuhata, T.: Proc. Japan Acad., **40**, 679 (1964).
- 2) Campbell, D. H., and Gemzell, C. C.: Science, **112**, 1091 (1955).
- 3) Hirschfeld, J.: Science Tools, **7** (2), 18 (1960).
- 4) Stanworth, D. R.: Nature, **188**, 156 (1960).
- 5) Chan, P. C., and Deutsch, H. F.: Immun., **85**, 37 (1960).