STUDIES ON IMMUNOGLOBULINS FROM NORMAL AND TOXOPLASMA-INFECTED RAT SERA ON DEAE-SEPHADEX A 50 IN MULTILAYER MICROCOLUMNS

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Based on their immunological characteristics, the immunoglobulins are designated IgG, IgA, IgM, IgD, and IgE. The last two have hitherto been demonstrated only in human serum^{4,10}). It has not been reported that IgG was separated completely from IgM in rat serum by using any single method.

Chromatography of serum and other fluids of animals with ion exchangers of DEAE type is one of the useful tools for medical and veterinary research. Recently, chromatography of swine immunoglobulins on DEAE-Sephadex A 50 in microcolumns was introduced by MARTINSSON and JÖNSSON⁹). In their report a method of stepwise elution with increasing ionic strength and at constant pH was found to be most suitable. Therefore, the method was so modified as to be applicable to the separation of IgG and IgM in rat serum.

On the other hand, as shown in previous reports^{11,15}), remarkable changes in serum protein of rats infected with *Toxoplasma gondii* were the increase in gamma-globulins and the decrease in albumin. Antibody titers, as measured by the dye test, began to appear by day 3 to 5, showing the first infection, and remained at a high level for a long time^{8,12,16}).

In consequence, this method of separation of IgG and IgM in serum was used for studying different types of antibodies in rats infected experimentally with *T. gondii*. Then an attempt was made to investigate the behavior of shift of cytoplasm-modifying antibody and the transmission of the antibody to the fetus from the mother.

MATERIALS AND METHODS

Toxoplasma-infected rats

A total of 56 adult female rats of the Wistar-Imamichi strain over 8 weeks of age were used in an experiment on *Toxoplasma* infection. They were inoculated intraperi-

toneally with approximately 1×10^7 parasites of the Beverley-Shimizu strain¹⁵⁾ and the RH strain obtained from the peritoneal fluid of mice at 3 days of infection. The inoculum had been prepared in the following manner. Mouse peritoneal fluid was centrifuged at 2,000 rpm for 15 minutes. The resultant sedimentation was washed two times with physiological saline solution. The precipitated parasites were resuspended in saline to such extent that, when counted by the Neubauer hemocytometer, it might contain approximately 1×10^7 organisms in 1.0 ml of the suspension.

Rats were fed the conventional rat standard diet for 2 weeks. Then they were examined and inoculated intraperitoneally with 1.0 ml of suspension containing approximately 1×10^7 parasites. They were examined 3, 5, 7, 21, and 56 days postinfection (p.i.). Eight adult female rats were crossed with male rats 56 days p.i. Three of them and their fetuses were examined during a period immediately before delivery. Other 4 rats and their newborn infants were examined 3, 7, 21, 35, and 56 days after delivery. An attempt was made to isolate parasites from the fetuses and the placenta of their mother. Antibody titers against *Toxoplasma* were measured by the Sabin-Feldman dye test¹³⁾.

Sera and antisera

Human and rat sera from healthy controls were used without dialysis. The antisera against whole human serum, IgG, IgA, and IgM used were supplied by the Behring-werke AB.

Rabbit anti-whole serum, anti-IgG and anti-IgM rat serum were prepared by the authors⁶⁾. While constant stirring ammonium sulfate was added to 40% saturation, using undiluted sera. Crude gamma-globulins were prepared by starch zone electrophoresis. The gamma-globulins were fractionated into 3 peaks by gel filtration of Sephadex G-200. The first peak was subjected twice to the same method of fractionation. The first half area of the first peak was identified as IgM by agar gel immunoelectrophoresis. The second peak was fractionated by DEAE-cellulose column chromatography, and then the first half area of the first peak was identified as IgG by ultracentrifugation and agar gel immunoelectrophoresis.

Column chromatography

Dry DEAE-Sephadex A 50 was allowed to swell in 0.1 M phosphate buffer at pH 6.5 which was stirred continuously. After 2 hours the gel was allowed to settle and the supernatants were removed. The gel was resuspended in 0.048 M phosphate buffer 3 times, and then packed in multilayer microcolumns. As shown in Table 1, eight stepwise elutions with increasing ionic strength up to 0.5 M normal saline and at constant pH in phosphate buffer were regularly used⁶⁾. Four multilayer microcolumns 160 mm in length and 8 × 40 mm in size were applied. Before a column was used, 10 ml of the initial buffer was passed through it at the same speed as that used for chromatography. A constant flow of 18.6 ml per hour through the column was achieved by an autoapparatus of liquid chromatograph, JLC-5 AUH type, manufactured by the Japan Electric Optical Laboratory (JEOL), Tokyo.

Analysis of eluates

Eluates were collected in a fraction collector and the protein concentrations deter-

Table 1. Elution Buffers in 8-Stepwise Method

Buffer number	I	II	III	IV	V	VI	VII	VIII
Phosphate buffer Mol. pH 6.5	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.200
Normal saline Mol.	—	0.07	0.10	0.12	0.15	0.20	0.25	0.500
Changing time in minutes		25	55	90	120	160	195	235

Chart 1. Chromatography of Human Serum by 8-Stepwise Elution from DEAE-Sephadex A 50

The curves represent the absorbances at 280 m μ as an original, at 280 m μ as a scale of 3 times original, and at 260 m μ as a scale of 3 times original. The fraction numbers of pools 1 to 10 represent the tubes analyzed.

Chart 2. Chromatography of Rat Serum on DEAE-Sephadex A 50

The same symbols are used as in Fig. 1.

mined at 280 m μ and 260 m μ in the liquid chromatograph of JLC-5 AUH type. Fractions of 1.0 ml each were collected and pooled, as shown in Charts 1 and 2. They were concentrated to 0.5 ml by vacuum dialysis before analysis was performed by immunoelectrophoresis, immunodiffusion, and polyacrylamid gel and cellulose acetate membrane electrophoreses^{7,17}).

Chart 3. Changes in Serum Proteins, Glycoproteins and Lipoproteins of Rats Infected with *T. gondii*

Remarks. N: Non-infected rats. T. P.: Total protein. Alb: Albumin.
P. B. P.: Protein-bound polysaccharides. T-Chol: Total cholesterol.

RESULTS

Human serum

Chart 1 shows the elution pattern after DEAE-Sephadex chromatography on 0.4 ml of human serum. Fig. 1 gives the results obtained from the immunoelectrophoretic and immunodiffusion analyses of concentrated pools 1 to 10 against anti-human serum. Judging from the test on specific antisera, pool 1 derived from the fractionation of human serum contained mainly IgG. Pool 6 contained IgA. The typical IgM line could not be recognized by immunoelectrophoresis, but it became apparent when specific anti-IgM serum was used, as shown in pool 9 in Fig. 2.

Table 2. Antibody Determination of Immunoglobulins in Rats Infected with *T. gondii*

Days after i nfection	Days after delivery	Mother			Fetus or infant		
		Sabin-Feldman dye test			Sabin-Feldman dye test		
		Whole serum	IgM (Pool 9)	IgG (Pool 1)	Whole serum	IgM (Pool 9)	IgG (Pool 1)
3		<1 : 4	—	—			
5		1 : 16	+	—			
7		1 : 256~ 1 : 1024	+	+			
21		1 : 256~ 1 : 1024	—	+			
56		1 : 256	—	+			
83	—1				1 : 64	non-examined	
87	3	1 : 256	—	+	1 : 64	—	+
91	7	1 : 256	—	+	1 : 64	—	+
105	21	1 : 256	—	+	1 : 64	—	+
119	35	1 : 256	—	+	<1 : 4~ 1 : 16	—	—
140	56	1 : 256	—	+	<1 : 4	—	—

Normal rat serum

The fractions and pools obtained from rat serum by chromatography are indicated in Chart 2. The results of the immunoelectrophoretic analysis of whole serum and pools 1 to 10 of rat serum are shown in Fig. 1. In pool 1, IgG was detected alone. A gamma-globulin line, which was proved to represent IgA in human serum, was demonstrated in pool 6. It was not similar to the IgG or IgM line. Pool 9 contained IgM, as well as human serum did. The typical IgM line was seen in immunodiffusion analysis using specific anti-IgM serum, as shown in Fig. 3.

Toxoplasma infected rat serum

Chart 3 shows changes in serum proteins, glycoproteins, and lipoproteins in *Toxoplasma*-infected rats on the 1st, 3rd, and 8th week following inoculation with *Toxoplasma*. An increase in gamma-globulins was apparent over a period from the 1st to the 8th week p.i. A moderate increase in beta-globulins was shown until the 8th week p.i. Serum albumin decreased in percentage over the 8th week p.i. Of the glycoproteins in the serum, albumin contaminated with alpha-1 glycoprotein decreased and alpha-2 and beta-glycoproteins increased in the 1st week. There was a tendency for gamma-glycoproteins and protein-bound polysaccharides to decrease in total amount in the 8th week p.i. Alpha-2 and beta-lipoproteins tended to decrease in the 8th week p.i.

The results of antibody determination of the immunoglobulin preparations are given in Table 2 and Fig. 4. In the sera of adult female rats infected with *T. gondii*, antibody activity was observed in IgM only in pool 9 on the 5th day p.i., indicating a titer of 1 : 16 in the dye test. As shown in Fig. 4, a line representing the IgM portion was observed on the 5th day p.i., although it could not be found on the 3rd day. On the 7th day p.i. the antibodies were shown in both IgM in pool 9 and IgG in pool 1. After that they were observed in IgG only over a period from the 21st to the 140th day p.i. The fetuses in mother rats on the 83rd day p.i. showed a titer of 1 : 64 in the dye test. It was impossible to determine the IgM and IgG fractions, because of the extremely small amount of serum. The antibody activities in the newborn infants were observed in IgG alone by the 21st day after delivery. The titer determined by the dye test, which was 1 : 64 on

the 3rd day after birth, showed a tendency to decrease gradually by the 35th day. No titer could be detected by the dye test on the 56th day after birth. No parasite of *T. gondii* was found in any fetus or the placenta of 3 rats examined on the 83rd day p.i.

DISCUSSION

The first object of this investigation was to reduce the sample size, to simplify the procedure of chromatography, and to separate IgM completely from IgG of blood serum by one preparative method. BAUMSTARK¹⁾ has shown that the electrophoretic movement and accordingly the positions of lines appearing by immunoelectrophoresis are almost the same in animal serum as in human serum. Therefore, the immunoelectrophoretic precipitates of rats might be identified by direct comparison with the known lines of human serum. It was, however, admitted that such comparison was not a proof for identity. In consequence, the authors tried to prove some major components of rat serum, using specific antisera, such as albumin, transferrin, alpha-macroglobulin, IgG and IgM, made by themselves⁶⁾.

To separate immunoglobulins, the anion exchanger Sephadex A 50 was most suitable because of its high protein-binding capacity, as compared with those of Sephadex G-200, DEAE-cellulose, and DEAE-Sephadex A 25, which had been examined by the authors in a basic study. Thus, it was possible to reduce the use of buffer solution for elution. The 8-stepwise elution was more suitable for isolation of proteins in 4 multilayer micro-columns than the gradient elution with a small column⁶⁾. The ionic strength was increased by adding normal saline to the buffer, instead of increasing the molarity of the phosphate buffer, in the same way as mentioned by MARTINSSON⁹⁾.

The complete separation of IgG and IgM in rat serum was performed very satisfactorily by using the elution mentioned above. This method was therefore suitable for achieving the antibody separation for studies on the kinetics of immunoglobulins. Thus the chromatographic method was easier to carry out than any of the other methods, ultracentrifugation, zone electrophoresis, and gel filtration, which are much complicated.

Some investigators^{2,5,11)} conducted immunoelectrophoretic studies to characterize the protein components of rat serum. None of them, however, adequately defined the actual nature of many of the proteins represented by precipitin arcs.

The dye-test antibodies appeared on the 5th day of infection for the first time, showing their activity in IgM. When specific-pathogen-free (SPF) Wistar rats were used, results obtained from the immunoelectrophoretic patterns of the sera hardly revealed a line representing the IgM portion on the 3rd day p.i., but exhibited the same line on the 5th day p.i. In consequence, it was necessary to describe the results reported here as indicating the presence of IgM antibody against *Toxoplasma* in rats on the 5th day p.i. On the 7th day p.i. the antibody activity was found in both IgM and IgG, the latter of which had been separated completely from the IgM fraction. After that, it was shown in IgG alone in rats harboring encysted toxoplasmas in the brain. These results, as well as those reported by other authors^{8,12,15}, indicate that antibodies against *Toxoplasma*, as detected by the IgM and IgG dye test, were produced in rats in response to the infection with peracute toxoplasmosis or with encysted or chronic toxoplasmosis.

The present studies were performed on fetuses *in utero* and newborn infants collected from rats with chronic toxoplasmosis possessing antibodies of IgG origin. In them, only IgG antibodies against *Toxoplasma* were demonstrated in the fetuses and infants, from which no parasites were isolated. Thus, the IgG antibodies contained in the serum of the fetuses and newborn infants could be accountable for the transplacental passage of

maternal IgG, since the presence of 19S antibodies in the infants was an evidence of acute Toxoplasma infection¹²⁾. IgG antibody was demonstrated only in fetuses and infants born from rats with chronic toxoplasmosis and in adult female rats with persistent Toxoplasma infection. Studies are now in progress to clarify the migration of toxoplasmas from infected female rats to their fetuses and newborn infants.

SUMMARY

Separation of human and rat IgG and IgM was achieved by anion exchange chromatography on DEAE-Sephadex A 50 with 4 multilayer microcolumns (8×160 mm), using such a small amount as 0.4 ml of sample. IgG and IgM in rat serum were separated completely without contaminating fractions 1 and 9, respectively.

The antibody activities against *Toxoplasma gondii* in rat serum were observed in IgM alone on the 5th day, in both IgM and IgG on the 7th day, and in IgG alone by the 140th day postinfection.

Fetuses and newborn infants from rats with chronic toxoplasmosis showed titers determined by the dye test. The titers remained by 3 weeks after birth, presenting a tendency to disappear until 5 weeks of age. These titers were in IgG alone.

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Multilayer Microcolumns によるトキソプラズマ感染 ラットの免疫グロブリンについて

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ラットを用いて感染症の病態生理を研究するとき、とくに抗体蛋白のうち IgM と IgG について、動態を検索する際は、それぞれが全く混在することなく、簡易迅速に分画し得ることが望ましい。そこで著者らは、カラムクロマトグラフィーを用いる血清蛋白分画法に改良を加え、Multilayer microcolumns (8×160 mm) 中に DEAE-Sephadex A50 を用い、塩濃度上昇による 8 stepwise、流速 18.6 ml/1 時間での分画法を考案した。その結果、ラット血清中の IgG と IgM は、容易かつ完全に分離された。

ついで、トキソプラズマ RH 株または Beverley-Shimizu 株 $1.0 \times 10^7 \sim 1.5 \times 10^7$ 個を、成熟 Wistar-Imamichi 雌ラットの腹腔内に接種し、血清蛋白、糖蛋白、脂蛋白の動態と、免疫グロブリンの推移を調べた。そしてまた、トキソプラズマ感染後 8 週以上を経たラットの胎児と新生子につ

いても、同様に検討を加えた。

その結果、血清総蛋白、糖蛋白結合糖質、総コレステロール量の増加、さらに α -グロブリン、 α_2 -グリコプロティン、 γ -グロブリン分画の増加などについて、1 および 3 週目の動態には、著変を認めたが、8 週目の変動は一般に軽度であった。

一方、免疫グロブリン推移では、感染後 5 日目には IgM に、1 週目では IgM と IgG 双方に、そしてその後は IgG のみに抗体活性が認められた。胎児および新生子の Dye titers では、ともに一定の抗体価を示し、生後 3 週目を境にして漸減し、5 週目で完全陰性となるものも現われ、8 週目では全例陰性となった。新生子の抗体活性は、すべて IgG のみに認められた。今回の実験における胎児からは、トキソプラズマの分離はできなかった。

EXPLANATION OF PLATES

PLATE I

- Fig. 1. Immuno-electrophoretic analyses of whole serum and serum pools 1 to 10 derived from human and rat sera by chromatography. Anode is on the right.

PLATE II

- Fig. 2. Immunodiffusion analyses of pools 1, 6, and 9 fractionated in human serum with specific anti-IgG, IgA, and IgM sera. Numbers 1 to 10: Pools 1 to 10 fractionated in human serum, respectively, and w: human whole serum.

PLATE III

- Fig. 3. Immuno-electrophoresis of pools 1 and 9 fractionated in rat serum with specific anti-IgG and IgM sera. Anode is on the right.

PLATE IV

- Fig. 4. Antibodies against Toxoplasma in rats infected with *T. gondii*. Anode is on the bottom.

Fig. 1.

Fig. 2.

Fig. 3.

Pool 1
Anti-rat whole serum
Rat whole serum
Anti-rat IgG
Pool 1

Pool 9
Anti-rat whole serum
Rat whole serum
Anti-rat IgM
Pool 9

* a.i. = after infection.