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An Auto-Immune Antibody and the Antibody Producing Cells on the Erythrocytes of Rats Experimentally Infected with *Toxoplasma gondii*[1)]

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トキソプラズマ感染ラットの赤血球自己抗体と抗体産生細胞について

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In rats infected with *Toxoplasma gondii*, humoral antibodies were shown to be found in only IgM on the 5th day postinfection. On the 7th day postinfection, antibodies were found in both IgM and IgG, and were later observed in IgG alone from the 3rd to above the 25th week postinfection.

The erythrocytes in the rats were examined by means of the direct Coombs test, and the results were positive from the 4th week postinfection, although the positive phenomenon was slight. Positivity was also proved by the eluate factor from the positive erythrocytes. The Coombs tests with anti-rat IgG for newborn infants from mother rats in the 7th week postinfection were negative in all cases until the 5th week after birth. In the anti-globulins, after the absorption of IgG the positive phenomenon on the erythrocytes disappeared. In consequence, the auto-immune antibody on the erythrocytes of the rats infected with toxoplasma was thought to be similar to antigenic substances with the immunoglobulin G alone.

Rosette forming cells, which were thought to be the autoimmune antibody producing cells on the erythrocytes and having auto-erythrocytes of the infected rats, were found in the thymus, lymph nodes and spleen. In the fractionations of lymphoid cells, the rosette forming cells were found in the highest percentage in the 1st band of thymocytes. The

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band contained mainly small and middle sized lymphoid cells. No positivity of surface immunoglobulin G or M was found in any rosette forming cells. The cells of the 1st band in the thymus and spleen were shown to be in remarkable agglutination and immunofluorescence with the anti-rat thymocyte serum. Before use of the anti-thymocyte serum, it was adsorbed with bone marrow cells. The authors, therefore, presumed that the rosette forming cells with auto-erythrocytes of the 1st band in the thymus and spleen might belong to the series of the thymus derived lymphoid cells.

In the preceding parts of this series of studies on experimental toxoplasmosis, the authors have reported on the pathophysiological observations of an acute and a chronic toxoplasmosis in mice and rats infected with *Toxoplasma* (*T.*) *gondii*.[14,23~27] In these experiments,[24] only very few parasites of the virulent strain of *T. gondii* are required to kill a mouse within 10 days. Using the same strain, a rat can survive inoculation with millions of the parasites, showing a slight body defense reaction in the acute stage, having a tendency to recover to a subclinical stage in the 8th week postinfection.[22] As noted in the authors' earlier reports,[25] the activity of glucose-6-phosphate dehydrogenase, and lactate production in the erythrocytes in toxoplasma-infected rats in subclinical cases showed slight tendencies to decrease in the 8th week postinfection, showing a slightly shorter survival time than for the non-infected rats. The authors observed that the morphomaintenance and function of the erythrocytes in subclinical cases of rat toxoplasmosis might probably be affected by the toxoplasma parasites.

Reports are available on such hematological changes, as hemolytic anemia, erythroblastosis fetalis, Hodgkin's disease or lymphoma with toxoplasma parasites in human clinical cases.[2~4,9,10,13,17,19]

In this experiment, the authors attempted to confirm whether the slightly shortened life span and the changes of the erythrocytes in toxoplasma-infected rats were caused by auto-immune antibodies on the erythrocytes by the extracorpuscular abnormalities, and to determine whether the auto-immune antibody producing cells are derived from the thymus or the bone marrow cells.

MATERIALS AND METHODS

Wistar-Imamichi strain female rats, aged 5 to 8 weeks, were used for all experiments. Rats were inoculated intraperitoneally with 1×10^7 parasites of the RH or Beverley-Shimizu[28] strain obtained from the peritoneal fluid of 2-day infected mice. To prepare the inocula, mouse peritoneal fluid was centrifuged at 2,000 rpm for 15 minutes, and the lower portion of the fluid was then washed two times with physiological saline. The organisms were resuspended in the saline solution and adjusted to contain approximately 1×10^6 organisms in 1.0 m*l* of the suspension.

Antibody titers to toxoplasma were examined by the dye test and indirect immunofluorescent methods.[12]

Erythrocytes in acid-citrate-dextrose (ACD) at 4 C were washed four times with phosphate buffer saline solution (PBS) by centrifugation at 1,500 rpm for 10 minutes, so that the supernatant liquid and buffy coat were carefully removed. An aliquot of this suspension in the PBS after the last washing was diluted to 2% of the PBS solution.

Before the Coombs tests, anti-rat sera were adsorbed with the erythrocytes from all rats used for the experiment. This served to demonstrate incomplete antibodies on the erythrocytes. As for judging pseudopositive or true positive phenomenon in the Coombs test, the eluate test by Weiner was used.[14]

The direct Coombs test,[14] using macroscopical and immunofluorescent examinations, was carrid out by mixing 0.1 m*l* of the washed 2% erythrocyte solution with sera of 0.1 m*l* each from rabbits immunized with the following sera; rat-whole serum, rat-globulins, rat-IgG and rat-IgM.[7] These anti-rat sera were conjugated with fluorescein isothiocyanate (FITC),[5] and were inactivated at 56 C for 30 minutes. They were then centrifuged at 1,000 rpm for one minute. One drop of the sedimentation was put on the slide glass, and was then judged by the agglutination.

Lymphoid cells in the bone marrow, thymus, lymph nodes and spleen in rats were separated by the method of Conray 400-Ficoll.[10] The lymphoid cells isolated by the Conray 400-Ficoll method were separated in bands 1, 2, 3 and 4 by the arabic gum density method.[11] They were mixed with the erythrocytes of rats infected with toxoplasma at 4 C for one hour. Then the positive cells, auto-immune antibody producing cells with four or more auto-erythrocytes around the rosette forming cell, were counted by the method of HORIUCHI under microscopical examination.[8]

Rabbit anti-rat thymocyte serum (ATS) was prepared in the following manner. Suspensions of thymocytes for each of three injections into rabbits were obtained by removing the thymus from ether-killed rats immediately after death and homogenizing it in a cold PBS, pH 7.3. The thymocytes were washed three times in fresh PBS by centrifugation at 1,000 rpm for 10 minutes at 4 C. Following each centrifugation, the thymocytes were scraped from the residual erythrocytes with a spatula. The percentage of viable cells immediately prior to injection into rabbits was estimated by use of a 1:20 dilution of 0.1% Trypan blue.[13] Viability of the thymocytes ranged from 90–95%. A PBS suspension of approximately 5×10^8 thymocytes was injected into the marginal ear vein of the rabbit in each of three injections 7 days apart. At the same time, thymocytes suspended in the complete adjuvant were injected intramuscularly. Seven days following the third injection, the rabbits were exsanguinated by cardiac puncture, the blood was allowed to clot, and the serum was collected after adsorption as described below and then frozen at −20 C. Serum from control rabbits (NRS) which received injections of PBS without cells was handled similarly. The ATS was inactiva-

ted by incubation at 56 C for 30 minutes and subsequently adsorbed once with sheep erythrocytes washed three times with PBS at 37 C for one hour to remove heterophile (Forssman) antibodies. A refrigerated adsorption was made overnight at 4 C using the washed rat erythrocytes (1 m*l* of packed cells per 15 m*l* of serum). Incubated adsorptions at 37 C for one hour with 1 m*l* of packed rat erythrocytes per 30 m*l* of serum were repeated until the sera showed no evidence of agglutination of the rat erythrocytes under the microscope. In order to produce the specific thymus derived lymphocyte serum, ATS was adsorbed with rat liver and bone marrow cells. The serum was conjugated with FITC, and was then filtered into screw cap vials and held frozen at −20 C until used. Rat thymocyte agglutination titers were determined by the modified method of SLOBODA.[5] Serial dilutions of ATS were made with PBS. A solution of approximately 8×10^6 thrice washed rat thymocytes (0.02 m*l*) was added to each tube containing 0.5 m*l* of fluid. Control tubes included rat thymocytes in normal rabbit serum, thymocytes in PBS, and rat erythrocytes in serial dilutions of ATS, NRS, and PBS. All tubes were incubated at 37 C and examined against a lighted background for evidence of agglutination at two hours.

RESULTS

Humoral and auto-immune antibodies in the infected rats

Toxoplasma antibodies used in the dye test, and the indirect immunofluorescent methods obtained in the rats are shown in Table 1. SF dye titers appeared from the 5th day to above the 25th week postinfection (p.i.). By the indirect immunofluorescent method, toxoplasma antibody was found in only the IgM on the 5th day p.i. On the 1st week p.i., antibodies were observed in both IgM and IgG, and were later observed in IgG alone from the 4th to above the 25th week p.i. Antibody activities in the newborn infants from mother rats in the 7th week p.i. were observed in IgG alone up to the 5th week after delivery. No parasites of *T. gondii* were found in any infants in this experiment.

By means of the direct Coombs tests the erythrocytes in the rats were examined, and the results were positive in the anti-IgG tests, both in the macroscopical and immunofluorescent examinations, from the 4th week p.i. (Figs. 1-3). Positivity was also proved by the eluate factor from the positive erythrocytes. The direct Coombs tests with anti-rat globulin for the newborn infants on the 1st week after delivery was positive, showing a high percentage of reticulocytes. Otherwise, both the tests with anti-rat IgG and with the eluate factor from the erythrocytes in the infants were negative in all cases until the 5th week after birth.

In the anti-globulins after the absorption of IgG, the positive phenomenon on the erythrocytes disappeared. In consequence, the auto-immune antibody on the erythrocytes of the rat infected with toxoplasma was thought to be similar to antigenic substances

Table 1. Humoral Antibodies in Sera and Coombs Antibodies on the Erythrocytes in Rats Infected with *Toxoplasma gondii*

	Preinfection	Weeks postinfection									
		*5/7	1	2	3	4 Mating	7 Parturition	8	10	12	over 25
Toxoplasma antibodies											
Mother rats	<4	32	256			4096		4096		4096	4096
SF Dye titers		64	256			<16		<16		<16	<16
IgM		<4	256			16000		4096		>4096	4096
IgG											
Infant rats											
IgM								<4	<4	<4	
IgG								1024	256	16	
Direct Coombs antibodies											
Mother rats											
Whole serum	−	−	−	−	±	+		+	+	+	+
Globulin	−				−	+		+	+	+	+
IgG	−					+		+	+	+	+
IgM	−					−		−	−	−	−
Eluate from RBC**	−					+		+	+	+	+
Infant rats											
Globulin								+	−	−	
IgG								−	−	−	
Eluate from RBC								−			

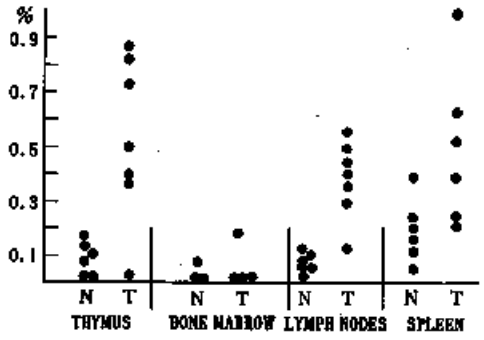
Notes: *5/7, 5 days postinfection; and **RBC, Erythrocytes.

with the immunoglobulin G alone.

Auto-immune antibody producing cells in the infected rats

As shown in Chart 1, rosette forming cells, these lymphoid cells with auto-erythrocyte clusters were separated by the Conray 400-Ficoll method. They were found in the thymus, lymph nodes and spleen in rats over 4 weeks p.i. Almost no cases of rosette forming cells were found in bone marrow.

In bands 1, 2, 3 and 4 of the cells resulting from the arabic gum separation, as



Notes: N, Normal rats; and T, Toxoplasma Infected Rats

Chart 1. Percentage of the Rosette Forming Cells in the Organes of Rats Infected with *Toxoplasma gondii*

Table 2. Specificities of the Cells in the Thymus, Lymph Nodes and Spleen of Rat Toxoplasmosis by the Arabic Gum Method

	Rosette forming cells (with autoerythrocytes)			Humoral Anti-sera (conjugated with FITC)						Anti-thymocyte serum (absorbed with bone marrow cells)					
				Anti-IgG			Anti-IgM			Fluorescent			Cell-agglutination		
	T	L	S	T	L	S	T	L	S	T	L	S	T	L	S
Bands 1	++	±	+	±	++	+	−	−	+	+	+	+	++	+	+
2	+	+	±	±	++	+	±	+	+	+	±	+	++	±	+
3	±	±	±	+	++	++	+	++	++	+	+	+	++	+	++
4	+	±	+	±	++	++	−	+	++	+	+	+	++	+	++

Remarks: T., Thymus; L., Lymph nodes and S., Spleen.
−, Negative; ±, Not decided as negative; +, Positive and ++, Strong positive.

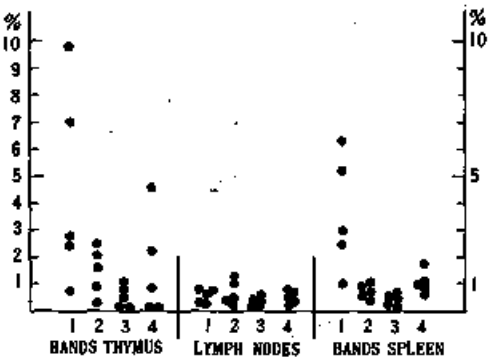


Chart 2. Distributions of the Rosette Forming Cells in Bands 1, 2, 3 and 4 by the Cell Separation in the Toxoplasma infected Rats

shown in Table 2 and Chart 2, the highest percentage of rosette forming cells in the organs was observed in the 1st band of the thymus and spleen, appearing mostly in lymphoid cells (Figs. 4-6). In the lymph nodes, the rosette forming cells were seldom found in bands 1, 2, 3 and 4.

By contrast, a high occurrence of the surface IgG-positive cells was found in bands 1, 2, 3 and 4 in the cell separation of lymph nodes and spleen. In the cells of the thymus, however, a low occurrence of the IgG-positivity was observed and that only in band 3. Surface IgM-positive cells in the spleen were found in bands 1, 2, 3 and 4, in bands 2, 3 and 4 of the lymph nodes, and in band 3 of the thymus cells.

As for the examination of specific ATS, all bands in the thymus and spleen cells, and bands 1, 3 and 4 in the lymph nodes were positive in the fluorescent and agglutination tests. No surface IgG- or IgM-positivity was found in rosette forming cells in any case.

Table 3. Rosette Forming Cells in the Thymus and Lymph Nodes of Rats Infected with Toxoplasma

No. Cases	Percentage of the rosette forming cells (Absorption with anti-thymocyte serum)			
	Thymus		Lymph nodes	
	Before	After	Before	After
1	20.3	11.3	2.5	2.3
2	15.4	2.7	1.5	0.4
3	14.3	1.3		
Average	16.7	5.1	2.0	1.3

When rosette forming cells in the thymus and lymph nodes were adsorbed with ATS, as shown in Table 3, the percentage of rosette formation decreased remarkably both in the cells of the thymus and in the lymph nodes.

DISCUSSION

In the present examinations for erythrocytes in rat toxoplasmosis by means of the direct Coombs test, the results were slightly positive in the anti-IgG tests, both in the macroscopical and immunofluorescent methods, from the 4th week postinfection. Positivity was also proved by the eluate factor from the positive erythrocytes. As for the indirect Coombs test in rats, since there were problems such as the pseudopositive phenomena resulting from isoantibodies, the authors did not use it in this experiment, although it is valuable in determining the serum factor which reacts as an incomplete antibody.

The authors, however, presumed that the positive results in the direct Coombs test are strong evidence for the presence of a so-called auto-immune hemolytic process. In its simplest interpretation this indicates the presence of an abnormal protein factor on the surface of the erythrocytes as reported in the preceding parts of this series of studies.[15,22~27] Immunologic changes in symptomatic or secondary hemolytic anemias have usually been identical with those found in the idiopathic forms.[4,20] Their frequent occurrence in diseases associated with abnormal proliferation of the reticuloendothelial system and lymphatic tissues has suggested to many investigators that the autoantibodies may be derived from these hyperplastic tissues.[29] Such antibodies have been extracted in high concentration from lymphosarcoma tissue[1] and the demonstration of a large amount of intracellular periodic acid-Schiff positive protein in the neoplastic tissue of malignant lymphomas, hemolytic anemia, lymphoma, Hodgkin's disease and positive antiglobulin tests has been interpreted as evidence that these tissues are capable of synthesizing abnormal proteins.[5,18] Toxoplasma parasites occasionally were found in these hematological diseases in human clinical cases.[2~4,9,10,17,19] In the authors past experiments, no remarkable changes in histopathological findings of rat toxoplasmosis were found in any of the experiments. However, although the mutual mechanism of the Coombs

positive phenomenon is not yet clear, it might be caused by the production of an incomplete antibody on the erythrocytes in rats after infection of toxoplasma. In the antiglobulins after the absorption of IgG, the Coombs positive phenomenon on the erythrocytes disappeared. The auto-immune antibody on the erythrocytes of the rat infected with toxoplasma was thought to be similar to antigenic substances with immunoglobulin G alone. The authros presumed that the slight changes of erythrokinetics in rat toxoplasmosis might be caused by the primary infection of toxoplasma. In consequence, the Coombs positive phenomenon might probably suggest the status of hematological changes, or latent auto-immune hematological disorder associated with toxoplasma infection.

On the other hand, rosette forming cells with auto-erythrocytes in rats infected with toxoplasma, which was thought as the auto-immune antibody producing cells on the erythrocytes,[8] were found in the thymus, lymph nodes and spleen. In the bone marrow, however, the cell was not found in quantity. In the fractionations of lymphoid cells by the method of arabic gum density gradient centrifugation,[11] the rosette forming cells were found in the highest percentage in the 1st band of thymus cells. The band contained mainly small and middle sized lymphoid cells. No positivities of surface immunoglobulins G and M were found in the rosette forming cells of the 1st band of the thymocytes even when the cells were agglutinated and made fluorescent by means of ATS. As for the auto-immune antibody producing cells in a typical auto-immune hemocyte anemia in human cases, it is known that the cell belongs to the abnormal plasmolytic series. In our present examination, however, the authors presumed that the rosette forming cells in rat toxoplasmosis may belong to the series of thymus derived lymphoid cells. In consequence, studies are now in progress to clarify whether the rosette forming cell shifts to the plasmocyte series.

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摘　　要

ヒトの Tp 症例のうちに，溶血性貧血，胎仔赤芽球症，ホジキン病，リンパ腫など血液疾患との併発として報告されているものがある。一方，ラットの不顕性 Tp 感染症時にも赤血球の形態・機能の面に異常をきたし，軽度ながら赤血球寿命の短縮，そして溶血し易い状態を呈することが知られている。これらの事から，著者らは Tp 感染時のラット赤血球について，主として赤血球自己抗体の面から検討を加えた。

その結果，Tp 接種後4週目から軽度ながら赤血球自己抗体の出現を認めた。陽性赤血球誘出試験の結果から，クームス陽性は血清蛋白等の非特異的吸着によるものでないことを知った。本陽性赤血球は抗 IgG とのみ陽性反応を示し，抗 IgG に対する特異抗体を吸収除去した抗グロブリン分画に対しては陰性を呈した。以上から赤血球自己抗体は IgG と同抗原性を有するものと考えた。

一方，自己赤血球ロゼット形成細胞，すなわち赤血球自己抗体産生細胞についての検討結果，胸腺，リンパ節および脾臓内細胞中に認められ，骨髄細胞中ではほとんど検出されなかった。アラビアゴム遠心分画より得たロゼット形成細胞の比率は胸腺および脾臓第 I 分画において最大で，主として小・中リンパ球様細胞と思われた。これらロゼット形成細胞を細胞膜表面 IgM および IgG 保有について検討したが，すべて陰性を呈した。特異ラット抗胸腺血清（肝・赤血球・骨髄細胞により吸収）とはきわめて強く反応した。これらの結果から，自己赤血球ロゼット形成細胞は，胸腺依存性リンパ球系に属するものと推察した。

Explanation of Plate I

Fig. 1. Negative agglutination in the direct Coombs anti-IgG test in non-infected rat erythrocytes.

Fig. 2. and 3. Positive agglutination in the direct Coombs anti-IgG test in toxoplasma-infected rat erythrocytes on the 4th week postinfection.

Fig. 4. A rosette forming cell with auto-erythrocytes in the 1st band of the thymocytes by the arabic gum method in a toxoplasma infected rat. Stained with May-Grunwald and Giemsa after 1% formaline fixation.

Fig. 5 and 6. Viable rosette forming cells with the auto-erythrocytes in the thymocytes of toxoplasma-infected rats. Stained with acridine Orange fluorrescence under the phase-contrast microscopical obeservation.

PLATE OMATA, Y. *et al.*