Changes in Distribution of T- and B-Lymphocytes in The Thymus, Spleen and Peripheral Blood of Normal and Toxoplasma-Infected Rats

Yoshitaka OMATA[2] and Naoyoshi SUZUKI[1]

(Department of Veterinary Physiology, Obihiro, University, Obihiro, Hokkaido, Japan)

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健康ラットおよびトキソプラズマ感染ラットの T-リンパ球と B-リンパ球について

小 俣 吉 孝*・鈴 木 直 義

Recent immunology has emphasized the unique function of thymus derived lymphocytes (T-cells) and bone marrow derived lymphocytes (B-cells) for cellular and humoral immune responses and co-operation between 2 lymphocyte subpopulations for the full expression of the immune response in experimental animals[6,7,10,19,21].

Evidence for the existence of T- and B-cells in man is also accumulating[11,42,43]. Ability to form rosettes directly under certain experimental conditions with sheep erythrocytes (E) is probably a T-cell property, as is mainly the ability to transform under stimulation of phytohemagglutinin in man. B-cells carry a high density of immunoglobulin on their surface[11,27] and can bind antigen-antibody complement complex through a membrane receptor for a modified component[37,45]. These cells can easily be identified by their ability to form rosettes with sheep erythrocytes which have heen sensitized with rabbit antibody against the Forssmann antigen and complement (EA (IgM) C). The EA (IgM) C will bind to the lymphocytes by the receptor for activated C' 3[22]. In recent reports concerning human diseases, it is said that changes of T- and B-cells in the peripheral blood may express specifically the status of the disease[16,33,36,38,40,43,45].

Immunological defence mechanisms of the toxoplasmosis are mainly caused by immune lymphocytes and macrophages in the cell mediated immunity[1,8,10,14,15,25,29]. At present, however, the role of immune lymphocytes in cellular immunity to infection

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2) Present address: Department of Protozoology, Research Institute for Microbial Diseases, School of Medicine, Osaka University, Yamada-Kami, Suita, Osaka.
帯広畜産大学家畜生理学教室

* 現住所：大阪府吹田市山田上，大阪大学微生物病研究所原虫学部

with the obligate intracellular protozoan *T. gondii* is not fully understood.

The authors here the results of an investigation designed to detect shifts in the ratio between two types of circulating lymphocytes in healthy and toxoplasma-infected rats. This research was undertaken since because no data existed concerning T- and B- lymphocytes in rats. The authors attempted to demonstrate whether rat lymphocytes from various lymphoid tissues can be divided into two subpopulations by means of their ability to bind E as T-cells and EA (IgM) C as B cells. As a result the relationship between the existence of rat thymocyte antigen or surface immunoglobulins by immunofluorescence and binding of E or EA (IgM) C in rat toxoplasmosis was clarified.

MATERIALS AND METHODS

Animals:

A total of 43 adult female rats, 6 to 8 weeks of age, of the Wistar-Imamichi strain were used as cell donors. Twenty-eight adult female rats were used in a toxoplasma infection experiment. They were inoculated intraperitoneally with approximately 1×10^7 parasites of the Beverley-Shimizu strain[31] and the RH strain obtained from the peritoneal fluid of mice at 2 days of infection. The inoculum was prepared in the same way as in our previous reports[34].

Rats were fed the conventional rat standared 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 prior to and 1, 3, 5, 7, 14, 21, 35 and 56 days postinfection. Antibody titers against toxoplasma were measured by the Sabin-Feldman dye test and the indirect immunofluorescent antibody test.

Cell collection from peripheral blood and cell suspension from lymphoid tissues:

Heparinized peripheral blood was allowed to sediment for an appropriate time and the leukocyte rich plasma was separated. Purification of lymphocytes was carried out by the method of Conray 400 Ficoll[39]. The lymphocytes isolated by the Conaay 400-Ficoll method were removed from the interface and the concentration was adjusted to 4×10^6 per ml in balanced salt solution (BSS).

The thymus, spleen and lymph nodes were minced into small pieces in 0.1% gelatin veronal buffer saline containing calcium and magnesium ion (GVB)[12], and passed through a steel mesh. Then the purification of lymphoid cells was also carried out by the Conray 400 Ficoll method. The lymphoid cell suspension thus obtained was washed three times by low speed centrifugation and resuspended and adjusted to 5×10^6 cells per ml in GVB. Viability of the cells was greater than 90%, and the percentage of viable cells was estimated by use of a 1:20 dilution of 0.1% trypan blue.

Sheep erythrocytes (E) and sheep erythrocyte-antibody-complement complexes (EA (IgM) C):

Sheep erythrocytes were stored at 4°C in ALSEVER's solution 1:1 and used until

hemolysis occured. Before use the cells were washed three times and adjusted to a 0.5 % suspension (approximately 80×10^6/ml) in BSS.

For the sensitization of sheep erythrocytes, the optimum amount of IgM rabbit antibody from sheep erythrocyte stroma[23] was used to avoid the interaction of lymphocytes with IgG antibody, if any. Subsequently 1×10^9 sensitized erythrocytes per ml of GVB were reacted with a 1 : 10 dilution of the mouse fresh serum at 37°C for 15 minutes, and then the erythrocytes were washed three times with GVB. EA (IgM) C cells were also adjusted to a cell concentration of 1×10^9 per ml.

Adherence of E or EA (IgM) C to lymphoid cells:

The same volume of E or EA (IgM) C suspension was mixed with 0.2 ml of lymphoid cell suspension (5×10^6 cells/ml) in small test tubes and incubated at 37°C for one hour in a water bath, then allowed to settle for a further hour at room temperature.

In some experiments, lymphoid cells were suspended in undiluted fetal calf serum (FCS) and reacted with E. Cell-free supernatant was discarded and the cells were resuspended in phosphate buffer saline (PBS) supplemented with 4 % bovine serum albumin. The cell suspension was smeared on a glass slide, dried quickly, and either stained with May-Grünwald and Giemsa or examined for immunofluorescence. The number of sheep erythrocytes which adhered to lymphoid cells was counted under the microscope. Smears were used to check the morphology of rosette-forming cells. Cells binding more than four erythrocytes were scored as a plus.

Demonstration of cell surface immunoglobulin determinants:

Living lymphocytes (5×10^5) from either rosette forming cells (RFC) or non-RFC populations were incubated with 0.05 ml of 1 : 20 diluted anti-rat immunoglobulin fluorescein conjugated at 4 C for one hour. Then they were washed twice with PBS, resuspended in a drop of 50 % glycerol-PBS and observed under a UV-microscope with or without phase-contrast (Photomax Universal Type, Olympus Co., Tokyo). More than 200 cells were counted and the percentage of the cells showing membrane attached fluorescence was recorded.

Immunofluorescent staining of thymocyte antigen:

Preparation of the anti-thymocyte serum (ATS) and the procedure of immunofluorescence have been described previously[24]. In order to produce the specific thymus derived lymphocyte serum, ATS was adsorbed with rat liver, erythrocytes and bone marrow cells. A cytotoxicity of ATS to lymphocytes was examined in the same sample which was stained with ATS immunofluorescence.

RESULTS

Distributions of B- and T lymphocytes in healthy rats:

As shown in Table 1 and Chart 1, percentages of human lymphocytes in peripheral blood having clusters of more than 4 EA (IgM) C and E were 40 and 57, respectively.

Table 1. Percentage of B- and T-lymphocytes from various tissues in healthy adult rats

Tissues	No. of tested	Percentage of lymphocytes					
		Bind E*	ATS	EA (IgM) C	Surf. Ig	ATS+ EA (IgM) C	ATS+Ig
Human							
Peri. blood	5	57.2±6.8**		40.4±5.4	38.7±2.1		
Rats							
Peri. blood	5	1.4±0.6	66.7±2.1	15.5±1.4	32.6±3.1	82.2±2.3	99.3±2.8
Lymph nodes	5	0.5±0.3	46.4±2.8	27.4±1.7	46.5±2.0	73.8±1.9	92.9±1.3
Thymus	5	1.3±0.6	87.1±1.8	2.1±0.5	3.1±0.7	89.2±0.7	90.2±1.6
Speeln	5	1.2±0.5	54.1±2.3	21.5±1.4	45.8±2.2	75.6±2.1	99.8±1.7

Remarks: B-lymphocytes were indicated by the test of EA (IgM) C and Surface Ig. T-lymphocytes were indicated by the test of E and ATS.

* Bind E, rosettes with sheep erythrocytes; ATS, anti-thymocyte serum positivity; EA (IgM) C, complement receptor of the sheep erythrocytes; and Surf. Ig, surface immunoglobulins.

** Mean ± S. E.

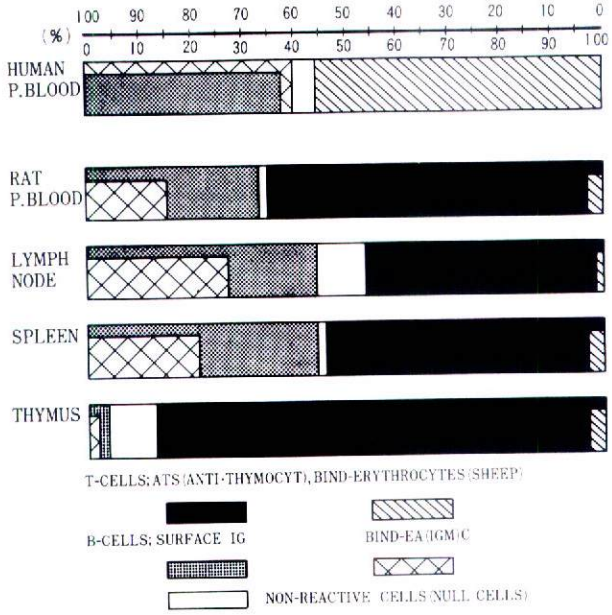

Chart 1. Populations of T- and B-lymphocytes in normal adult rats

In healthy rats, approximately 16 %, 22 % and 27 % lymphoid cells in the peripheral blood, spleen and lymph nodes formed rosettes as B-cells. In the thymus the percentage of thymocytes found in the rosette forming cells was small. Surface Ig was positively stained in the cytoplasm of lymphocytes and lymphoid cells, showing 32.6 % in peripheral blood, 46.5 % in lymph node cells, 3.1 % in thymocytes and 45.8 % in spleen cells.

As for the relationship of EA (IgM) C rosette forming lymphocytes to surface Ig-

positive cells, the Ig-positive cells were positively stained in the cytoplasm of almost all lymphocytes which formed rosettes with EA (IgM) C. Although the percentage of surface Ig-positive cells was greater than EA (IgM) C rosette-forming cells, there was a similar tendency in percentage between these two markers.

Approximately 57% of human lymphocytes from peripheral blood had clusters of more than 4 sheep erythrocytes (E) and formed rosettes. However, very few thymocytes, lymphoid cells and lymphocytes in rats clustered with sheep erythrocytes. ATS against thymocytes showed a positive staining of 87% by immunofluorescence. The lymph nodes and spleen of rats contained 46.4% and 54.1% of cells staining weakly for ATS. Lymphocytes in peripheral blood against ATS showed 66.7%.

As for the relationship of surface Ig-positive lymphocytes to ATS-positive cells, the Ig-positive cells as B-cells were almost always ATS-negative. Only a few Ig-positive lymphocytes were observed to show diffusely ATS-positive. Therefore, ATS-positive cells seem to be independent from surface-Ig positive cells. From these results in healthy rats, the methods of ATS for T-lymphocytes and surface Ig for B-lymphocytes were suitable for use, showing nearly 100% in total positive lymphocytes or lymphoid cells.

Distribution of ATS-positive and surface Ig-positive cells in rats infected with Toxoplasma gondii:

As shown in Tables 2 and 3 and Chart 2, ATS-positive cells in the rat thymus

Table 2. Percentage of T-lymphocytes from various tissues which bind E and ATS positive in rats infected with *T. gondii*

Tissues	No. of tested	Percentage of lymphocytes in rats (days postinfection) 7		56	
		Bind E Mean±SE	ATS positive Mean±SE	Bind E Mean±SE	ATS positive Mean±SE
Peripheral blood	6	0.7±0.4	63.1±2.5	0.9±0.5	56.7±2.6
Lymph nodes	6	0.3±0.2	46.0±2.1	0.2±0.3	45.7±2.7
Thymus	6	0.4±0.3	92.7±1.5	0.4±0.3	90.4±1.7
Spleen	6	0.3±0.5	68.4±2.2	0.4±0.3	46.1±2.5

Table 3. Percentage of B-lymphocytes from various tissues which bind EA (IgM) C and Surface Immunoglobulins in rats infected with *T. gondii*

Tissues	No. of tested	Percentage of lymphocytes in rats (days postinfection) 7		56	
		EA (IgM) C Mean+SE	Surface Ig Mean+SE	EA (IgM) C Mean+SE	Surface Ig Mean+SE
Peripheral blood	6	7.3±0.9	27.9±1.9	18.1±1.6	24.8±2.5
Lymph nodes	6	11.4±1.4	43.1±2.5	48.1±2.6	45.4±2.2
Thymus	6	4.8±0.9	3.3±0.9	2.7±0.1	1.4±0.1
Spleen	6	15.0±1.3	24.9±1.7	25.6±1.9	37.1±2.3

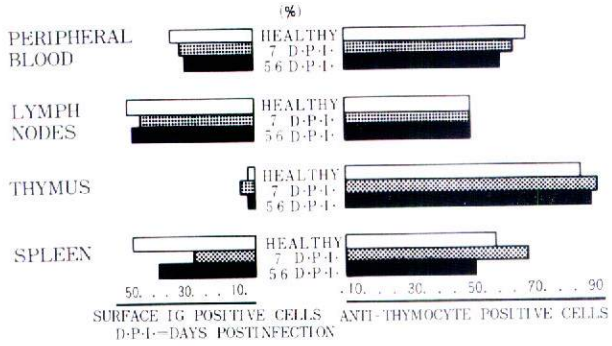

Chart 2. Percentage of lymphocytes in blood of toxoplasma infected rats

infected with Toxoplasma had increased to 92.7 on the 7th day and 90.4% on the 56th day p.i. as compared with 87.1% in healthy rats. Most of these cells in the thymus were strongly fluorescing small lymphocytes. The remainder were large or medium-sized cells which showed weak but clear-cut fluorescence. The spleen contained 68% ATS-positive of weakly fluorescing small lymphocytes on the 7th day, falling to 46% on the 56th day p.i. T-cells in the lymph nodes of rats infected with Toxoplasma were found to increase on the 7th day p.i. and decrease on the 56th day p.i.

As for B-cells identified through the EA (IgM) C and surface Ig tests, a remarkable decrease in the spleen was observed on the 7th day p.i., tending to recover to the percentage of healthy rats. In the thymus, a slight increase of B-cells was found on the 7th day p.i.

In the peripheral blood as shown in Table 4, the lymphocyte count decreased to 6.1 thousand per cubic milimeter of blood on the 7th day p.i., having been 8.5 thousand before infection. The value of lymphocytes showed an increase on the 56th day p.i. The percentage of T-cells in peripheral blood decreased gradually, showing a percentage of 62.1 on the 7th day and 56.7 on the 56th day p.i. as compared to a value of 66.7% before infection. The Ig-positive lymphocytes also showed a similar tendency to decrease gradually.

Table 4. Absolute Counts and Percentage of Lymphocytes in Peripheral Blood of Toxoplasma-infected Rats

Donor	No. of animals	Lymphocyte count 10^3 Mean ± S.E.	ATS positive % Mean ± S.E.	Ig positive % Mean ± S.E.	Total positive cells % Mean ± S.E.
Healthy rats	10	8.5±1.4	66.7±2.1	32.6±3.1	96.3±2.8
Toxoplasma infected rats (days postinfection)					
7	6	6.1±1.3	62.1±2.5	27.9±1.9	99.5±4.9
56	6	9.8±1.1	56.7±2.6	24.8±2.5	81.3±3.1

DISCUSSION

It is well documented in mice that there exist at least two functionally different subpopulations, T-cells and B-cells, and that they are characterized by appropriate cell markers[17,27]. It has also been reported that some human lymphocytes bind sheep erythrocytes to form rosettes as T-cells[2] and that some human lymphocytes bind antigen-antibody-complement complexes[3].

The quantitation of T- and B-cells of rat lymphocytes by the tests of the positivities of anti-thymocyte serum and of surface immunoglobulins was compared with that of the conventional methods, E-binding cells and EA (IgM) C-binding cells, in human lymphocytes.

In all cases, percentage of total positive lymphocytes reached approximately a hundred per cent in healthy rats. This means that rat lymphoytes or lymphoid cells belong to either T-cell or B-cell subpopulations. T-cells increased slightly in the lymph nodes, spleen and thymus in rats on the 7th day postinfection of Toxplasmas.

The most original feature of the primary response to Toxoplasma in the rat was a sharp peak of IgM and the appearance of IgG humoral antibodies on the 7th day postinfection[34,35]. In recent works by the authors[30,32], Toxoplasma parasites were mainly taken up by macrophages, some then passing on to lymphocytes or lymphoid cells leading to activated T-cells and B-cells. B-cells differentiate into antibody synthe sizing cells often interacting subsequently with activated T-cell[41]. Consequently, areactive increase of T-cells in the spleen may have been caused by the requirement of the co-operation of T-cells acting as helper cells leading in some way to an immune response by the B-cells.

It is yet unknown whether the changes of the individual behaviour of particular cell populations are produced by the direct effect of the defence mechanisms to the Toxoplasma parasite. Further experiments should be performed to clarify the immune response, a highly complex process involving finely balanced inter-relationships between many different types of T- and B-cells resulting from the behaviour of the central lymphoid organ such as the thymus.

SUMMARY

As for the detection of B- and T-lymphocytes in healthy adult rats, the methods of ATS for the T-cells and of surface Ig for the B-cells were suitable for use, showing nearly a hundred per cent in total positive lymphocytes or lymphoid cells in the peripheral blood, spleen, lymph nodes and thymus.

In the variation of the T-cells and B-cells in rats infected experimentally with *T. gondii*, an increase of the T-cells and a decrease of B-cells were found in the spleen on the 7th day postinfection.

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摘　　要

最近，宿主生体内免疫応答を担当するリンパ球は大別して2種類あることがわかって来た。すなわち，T-細胞とB-細胞が，それである。

一般に，ヒトでは羊赤血球(E)に対しロゼットを形成する細胞(T-細胞)と，補体結合感作羊赤血球(EA (IgM) C)に対しロゼットを形成する細胞あるいる表面免疫グロブリン(Ig)陽性細胞(B-細胞)に分別している。

胸腺依存性(Thymus-dependent)リンパ球をT-細胞と定義することから，特異抗胸腺細胞家兎血清(ATS)との細胞障害性および特異蛍光抗体法による陽性細胞がT-基幹細胞であると考えられている。そこで，これらの方法を用いてラットリンパ球の分別を試みた。

その結果，健康ラットの末梢血液，腸間膜リンパ節，胸腺および脾臓内リンパ球のうちATS陽性細胞は66.7，46.4，87.1，54.1%で，Ig陽性細胞は32.6，46.5，3.1，45.8%であった。羊赤血球とのロゼット形成細胞は，ほぼ1%内外でラットのT-細胞分別法としては用いられない。一方，EA (IgM) C法によるB-細胞分別ではIg法と百分率で平行するが，いわゆる，Null細胞がIg法に比して多い。したがって，ラットのT-細胞とB-細胞の分別にはATSとIg陽性細胞の検出法により，その目的を達しうると考えた。

トキソプラズマ原虫(Tp)感染に伴うリンパ球数の変動では，感染初期における脾臓内T-細胞の相対的増加とB-細胞の減少が特徴的であり，かつまた非反応(Null)細胞が増加した。