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Abstract

Human peripheral blood mononuclear cells (PBM) were separated into sheep erythrocyte rosette-forming (Es+) and non Es+ cells by the Ficoll-Hypaque gradient sedimentation method. Thirty-eight percent of the Es+ cells formed rosettes with dog erythrocytes and were designated as Es+Ed+ cells. The remaining Es+ cells were designated as Es+Ed- cells. Only a few non Es+ cells formed rosettes with dog erythrocytes. Among Es+Ed+ cells, T4 antigen-positive cells were observed approximately 1.7 times as often as T8 antigen-positive cells, when measured by staining with OKT4 or OKT8 monoclonal antibody. Among Es+Ed- cells, however, T4 and T8 antigen-positive cells were observed in almost equal proportion. Preincubation of PBM with OKT11 monoclonal antibody, but not with OKT4 monoclonal antibody, inhibited the rosette formation with dog as well as sheep erythrocytes. These results indicated that Es+Ed+ cells were a subpopulation of T-cells in which a majority of the cells were T4 antigen-positive, and that the binding sites of dog erythrocytes on human T-cells was closely linked with that of sheep erythrocytes.

KEYWORDS: dog erythrocyte, rosette formation, T-cells, E-receptor, OKT11

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Dog Erythrocyte Rosette-Forming Lymphocyte: Blockage by OKT11 Monoclonal Antibody

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Human peripheral blood mononuclear cells (PBM) were separated into sheep erythrocyte rosette-forming (Es^+) and non Es^+ cells by the Ficoll-Hypaque gradient sedimentation method. Thirty-eight percent of the Es^+ cells formed rosettes with dog erythrocytes and were designated as Es^+Ed^+ cells. The remaining Es^+ cells were designated as Es^+Ed^- cells. Only a few non Es^+ cells formed rosettes with dog erythrocytes. Among Es^+Ed^+ cells, T4 antigen-positive cells were observed approximately 1.7 times as often as T8 antigen-positive cells, when measured by staining with OKT4 or OKT8 monoclonal antibody. Among Es^+Ed^- cells, however, T4 and T8 antigen-positive cells were observed in almost equal proportion. Preincubation of PBM with OKT11 monoclonal antibody, but not with OKT4 monoclonal antibody, inhibited the rosette formation with dog as well as sheep erythrocytes. These results indicated that Es^+Ed^+ cells were a subpopulation of T-cells in which a majority of the cells were T4 antigen-positive, and that the binding sites of dog erythrocytes on human T-cells was closely linked with that of sheep erythrocytes.

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It has been recognized for more than 10 years that human T lymphocytes form rosettes with heterologous erythrocytes from sheep (1), dogs (2), goats (3), horses (4), pigs (5), humans (6), rabbits (7) and monkeys (*rhesus*) (8), and that human B lymphocytes form rosettes with erythrocytes from mice (9) and monkeys (*Macaca speciosa*) (10). The rosette formation has been used as a surface marker of human lymphocytes, *e.g.*, rosette formation with sheep erythrocytes as a T cell marker and that with mouse erythrocytes as a B cell marker. Recent advances in cell engineering techniques have made it possible to produce mouse monoclonal antibodies (mAb) which

react with multiple epitopes on human lymphocytes. A mAb binds to a specific antigen on human T-cells to define different functional T-cell subsets, *e.g.*, OKT4 for helper/inducer and OKT5/8 for suppressor/cytotoxic T-cells (11).

It is speculated that heterologous erythrocytes from various animals might bind to different antigens expressed on a certain subpopulation of human lymphocytes or T-cell subsets. If so, rosette formation with heterologous erythrocytes from different species would provide a useful method of classifying functional T-cell subsets.

In this study, we examined a subpopulation of dog erythrocyte (Ed) rosette-forming

cells by separation of peripheral blood mononuclear cells into T- and non T-cells and staining with OKT4 and OKT8 mAb.

Materials and Methods

Human peripheral blood mononuclear cells (PBM) were isolated from heparinized venous blood of 9 healthy persons on our laboratory staff by Ficoll-Hypaque gradient sedimentation as described previously (2). The cells were washed 3 times and suspended at 5×10^6 cells/ml in RPMI 1640 medium (GIBCO Laboratories, Life Technologies Inc., Ohio, U. S. A.) supplemented with 2 mM L-glutamine, 100 μ g/ml of streptomycin, 100 u/ml of penicillin G and 10% fetal calf serum (FCS, GIBCO, U.S.A.) heat-inactivated and preabsorbed with sheep and dog erythrocytes. Sheep erythrocytes (Es) in Alsever solution were supplied by West Japan Sheep Farm Company. Dog erythrocytes (Ed) were obtained from heparinized venous blood drawn from mongrel dogs. Es and Ed were washed 3 times in phosphate-buffered saline (PBS; pH 7.2, 0.1 M) and suspended at a concentration of 1% (v/v) in RPMI 1640 medium.

One hundred microliters of PBM suspension was mixed with the same volume of Es or Ed suspension, incubated at 37°C for 10 min, and centrifuged at $180 \times g$ for 5 min. The precipitated cells were gently suspended in the medium, and at least 200 cells were counted in a haemocytometer under a light microscope. Lymphocytes to which three or more erythrocytes were attached were judged to be rosette-forming cells (RFC).

The method for isolation in Es-RFC (Es^+) and non Es-RFC (Es^-) was described elsewhere (12). Briefly, suspensions of PBM and Es were mixed after the rosetting procedure and layered on Ficoll-Hypaque solution and centrifuged at $400 \times g$ for 30 min. Es^- cells were collected from the interphase layer and Es^+ cells were obtained from the bottom of the test tube. Es^+ cells were rosetted again with Ed after the lysis of Es in 0.83% NH_4Cl solution. After overnight incubation, the Es^+Ed^+ cell population was separated from the Es^-Ed^- cell population by Ficoll-Hypaque gradient sedimentation, and Ed were lysed in the

same way as Es.

Mouse mAbs which reacted with human helper/inducer T-cells (OKT4), suppressor/cytotoxic T-cells (OKT8) and E-receptor on T-cells (OKT11) were purchased from Ortho-mune, Ortho Diagnostic Systems Inc., N. J., U. S. A. One million Es^+Ed^+ or Es^+Ed^- cells were incubated at 4°C for 60 min with 25 μ l of mouse mAb diluted 5 times with PBS, and incubated at 4°C for 60 min with 25 μ l of goat anti-mouse immunoglobulin antibody labelled with FITC (Ortho, U. S. A., $\times 5$ dilution). After washing 3 times with PBS, the cells were mounted in glycerol buffer and observed under an immunofluorescence microscope (Olympus, Japan).

Results and Discussion

Previously, we found PBM which formed rosettes with both sheep and dog erythrocytes (Es and Ed) (2). Es and Ed can easily be distinguished from each other by size, because Ed are larger than Es. Moreover, the study showed that most of the dog erythrocyte rosette-forming cells (Ed^+ cells) belonged to a subpopulation of sheep erythrocyte rosette-forming cells (Es^+ cells) as shown in Table 1. Thirty-eight percent of the Es^+ cells (T-cells) were Ed^+ cells (Table 1). Thus, Ed^+ cells

Table 1 Percentages of human lymphocytes forming rosettes with sheep erythrocytes (Es) or with dog erythrocytes (Ed)

Attached erythrocytes	% Rosette-forming cells (RFC) ^a among		
	PBM ^b	Es-RFC ^c	non Es-RFC ^d
Es	65 \pm 4	77 \pm 5	14 \pm 6
Ed	40 \pm 14	38 \pm 13	7 \pm 3

a: Each value represents the Mean \pm S. D. of 9 experiments.

b: PBM, peripheral blood mononuclear cells.

c, d: Sheep erythrocyte rosette-forming cells (Es-RFC) and non Es-RFC were separated from PBM after rosette formation by Ficoll-Hypaque gradient sedimentation, and Es attached to lymphocytes were lysed in 0.83% NH_4Cl solution. Then, cells were rosetted again with Es or Ed.

Table 2 Percentage of OKT4 and OKT8 positive cells in Es^+Ed^+ and Es^+Ed^- cell populations^a

Experiment Number	Cell population	Percentage of cells positively stained with		Ratio of T4- to T8-antigen positive cells (T4/T8)
		OKT4	OKT8	
1	Es^+Ed^+	44	29	1.52
	Es^+Ed^-	32	39	0.82
2	Es^+Ed^+	53	40	1.33
	Es^+Ed^-	27	28	0.96
3	Es^+Ed^+	64	30	2.13
	Es^+Ed^-	43	39	1.10

a: Sheep erythrocyte rosette-forming cells (Es^+ cells) were lysed and rosetted again with dog erythrocytes (Ed). Cells forming rosettes with both sheep and dog erythrocytes (Es^+Ed^+) were separated from cells forming rosettes with only sheep erythrocytes (Es^+Ed^-) by Ficoll-Hypaque gradient sedimentation. Ed attached to lymphocytes were lysed in 0.83% NH₄Cl solution before staining with monoclonal antibodies (OKT4 and OKT8).

were a T-cell subpopulation. In order to examine which antigens are expressed on the cell surface of Ed^+ cells, Es^+Ed^+ cells were separated from Es^+Ed^- cells by Ficoll-Hypaque gradient sedimentation and stained with OKT4 and OKT8 mAb. The results are shown in Table 2. The majority of the Es^+Ed^+ cells had T4 antigen, and the ratio of T4 to T8 antigen positive cells (T4/T8) ranged from 1.33 to 2.13. On the other hand, an almost equal percentage of Es^+Ed^- cells had either T4 or T8 antigens, and the T4/T8 ratio ranged from 0.82 to 1.10.

Human T-cells with T4 antigen are known to function as a helper of immunoglobulin (Ig) production by B-cells (11). We examined the helper effect of Es^+Ed^+ and Es^+Ed^- cells on *in vitro* Ig synthesis by human B-cells. B-enriched cells (non Es -RFC, 7.5×10^5 cells) were cocultured with Es^+Ed^+ or Es^+Ed^- cells (2.5×10^5 cells) in the presence of 10 μ l/ml of pokeweed mitogen (DIFCO) for 6 days. The amount of IgG in the culture supernatant

was more when B-enriched cells were cultured with Es^+Ed^+ cells than when they were cultured with Es^+Ed^- cells, when measured by enzyme-linked immunosorbent assay (data not shown). Es^+Ed^+ cells in which T4 antigen positive cells were a predominant population showed more helper function on B-cells than Es^+Ed^- cells.

Active E rosette-forming cells (active ERFC) are known to be a T-cell subset which possesses a high affinity for Es . Chassagne *et al.* (13) showed by using active ERFC prepared by Ficoll-Hypaque gradient that the majority of the active ERFC was OKT8 antigen positive (T4/T8 ratio: 0.74). Es^+Ed^+ cells may be a different subset of T-cells from the active ERFC, because the majority of Es^+Ed^+ cells was OKT4 antigen positive, as reported in the present paper.

Munker *et al.* (7) reported that rabbit red blood cell (Er) rosette-forming cells were thymocytes which expressed antigens of immature thymocytes (OKT6) and mitogen activated peripheral lymphocytes. Ed

Table 3 Inhibition of Es - and Ed -rosette formation by OKT11 monoclonal antibody^a

Experiment Number	Antibody preincubated	Percentage of cells forming rosettes with	
		Es	Ed
1	PBS (control)	39	11
	OKT11	1	0
2	PBS	51	26
	OKT4	52	25
3	OKT11	1	1
	PBS	67	15
	OKT4	66	18
	OKT11	45	6

a: One million peripheral blood lymphocytes were preincubated at 4°C for 1 h with 25 μ l of phosphate-buffered saline (PBS) or monoclonal antibodies (diluted 5 times with PBS). The cells were washed 3 times with PBS and mixed with sheep erythrocytes (Es) or dog erythrocytes (Ed). The percentage of rosette forming cells was calculated as described in Materials and Methods.

rosette-forming cells, on the other hand, were found among unstimulated PBM. They also reported that OKT11 mAb, which is known to inhibit Es rosette formation by binding to the Es-receptor molecule (T11), inhibited Er-rosette formation (14, 7). We found that OKT11 mAb, but not OKT4 mAb, inhibited both Es and Ed rosette formation (Table 3). Hünig recently demonstrated that OKT11 mAb inhibited Eh (human red blood cells) rosette formation as well as Es rosette formation, and that the mAb against the target structure for T11 molecules (T11 TS) on sheep erythrocytes inhibited Es but not Eh rosette formation (15).

To find out whether or not Es- and Ed-receptors are the same, purification and comparison of the receptor molecules will be necessary. Under the present knowledge, it may be said that receptors of human T-cells for heterologous erythrocytes from various animals such as sheep, dogs, rabbits and humans are closely-linked because their rosette formations were commonly inhibited by OKT11 mAb. However, the binding sites might be different, because Es-, Ed- and Eh-receptors are expressed on unstimulated lymphocytes while the Er-receptor is expressed on activated lymphocytes (1, 2, 6, 7) and because mAb against T11 TS inhibits Es but not Eh rosette formation. It is also possible that molecules with different antigenicity on Es or Eh bind to the same receptor (T11).

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