Erythrocyte-Rosetting Properties of Feline Blood Lymphocytes and Their Relationship to Monoclonal Antibodies to T Lymphocytes

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Rosette formation of feline peripheral blood leukocytes with guinea pig (GP) and gerbil (G) erythrocytes (E) has been shown in an earlier study to identify T lymphocytes expressing helper and suppressor cell activity, respectively. This T lymphocyte distinction was based on the removal of the E-rosetting populations from peripheral blood leukocytes (PBL) and the subsequent functional evaluation of the remaining cells in a pokeweed mitogen (PWM)-induced synthesis of immunoglobulin (Ig). In the present study, we demonstrate a direct helper and suppressor function of GPE- and GE-rosetted cells, respectively, wherein the induction of Ig synthesis is altered in a positive or negative way by the addition of the cells to a control target population. A pan-T monoclonal antibody (mAb), CT843, and mAbs to the CD4 (CT248) and CD8 (CT87) subsets are also described; their specificities are established in functional assays, the PWM-induced Ig synthesis and the production of interleukin-2 following Concanavalin A stimulation of PBL, and a biochemical analysis of the surface membrane antigens detected by the mAbs. Immunoprecipitation and SDS-PAGE analyses showed CT248 to react with a ~60-kDa protein under both reducing and nonreducing conditions. Under reducing conditions, CT87 reacted with one subunit at ~35 kDa; a second faint band at ~39 kDa was poorly resolved. mAb CT843 detected a heterodimer of ~70 and ~60 kDa under both reducing and nonreducing conditions. The relationship of the mAbs to E-rosetting was examined in FACScan analyses and rosette inhibition studies. The percentage of GE-rosetting cells agreed with the percentage of cells stained with the CD8 mAb, whereas a comparison of GPE-rosetting and staining with the CD4 mAb showed variability. The binding of GE to PBL was blocked by pretreatment of PBL with the CD8 mAb, whereas no inhibition of GPE rosettes was observed with any of the mAbs. In a previous study, we had shown that an overnight culture of feline PBL at 37°C leads to the development of a second population of GPE-rosetting cells, also having a helper function. The relationship of the two GPE-rosetting populations to the CD4 mAb, CT248, was examined in rosette depletion studies and FACScan analyses. It was found that depletion of the GPE-rosetting cells from fresh, i.e., Day 0 cells, removed only a small percentage of cells reactive with the CD4 mAb, whereas GPE-rosette depletions performed on Day 1 PBL, which contained both populations of GPE-rosetting cells, removed almost all cells reactive with this antibody. The latter study suggests that the GPE-rosetting phenomenon is detecting two subsets of CD4 cells with T helper function, those present in fresh blood and those acquiring the GPE receptor after an overnight culture. Exp Biol Med 227:771–778, 2002

Key words: rosettes; T lymphocytes; feline; monoclonal antibodies.

Monoclonal antibodies identifying feline T lymphocytes (1–3), B lymphocytes (4), and monocytes (5) have been reported by investigators using this species as an animal model for a variety of clinical diseases in humans. Prior to the availability of these reagents, however, other more classical laboratory methodologies were used, e.g., erythrocyte (E) rosetting for T cells, surface immunoglobulin (Ig) detection for B cells, and the nonspecific esterase stain for monocytes. Early studies had suggested that guinea pig (GP) E were rosetting with T lymphocytes of the cat, a finding analogous to the rosetting of sheep E used to detect T lymphocytes in humans (6–8). In examining the E-rosetting properties of feline lymphocytes, however, we reported a rather novel finding for this species, i.e., an apparent helper cell activity for GPE-rosetting cells and a suppressor function for lymphocytes rosetting with gerbil (G) E (9, 10). The E-rosetting T lymphocyte distinctions were revealed in assays for Ig synthesis and interleukin (IL)-2 production following removal of the E-rosetting populations from the defining reaction systems, polyclonal activation of peripheral blood leukocytes (PBL) with pokeweed mitogen (PMW) and Concanavalin A (ConA). The E-rosetting phenomenon with GPE was compounded fur-

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ther by the observation that an incubation of blood lymphocytes overnight at 37°C yielded a second population of GPE-rosetting cells, also shown to express helper activity (10). Although it is recognized that E-rosetting as a methodology to identify and quantitate T lymphocytes has been supplanted by monoclonal antibody technology, the rather unique rosetting properties expressed by feline lymphocytes prompted an extended analysis of this novel phenomenon. The objective of this study was to verify the apparent T cell subset distinctions made by G and GP E rosetting, and to determine the relationship of the rosetting cells expressing T helper (T-H) and T suppressor (T-S) activities to the respective feline CD4 and CD8 subsets identified by monoclonal antibodies to lymphocyte cell-surface antigens.

Methods

Animals. Young (1–3-year-old), healthy SPF adult cats were used. This study was approved by the University of Tennessee's Animal Care and Use Committee of the Veterinary College in accordance with National Institutes of health Guidelines and Federal Law.

Tissue. Blood was defibrinated on a shaker with glass beads. The PBL were separated on a Ficoll-Hypaque gradient, and after washing with incomplete Hanks' balanced salt solution (iHBSS), they were suspended in RPMI-1640 with 10% fetal calf serum (FCS) that had been absorbed with sheep, GP, and G red blood cells (RBC). Thymic cells were obtained from 8- to 10-week-old kittens; the cells were cryopreserved in liquid nitrogen with dimethyl sulfoxide and were recovered for testing when desired.

Rosette Analyses: Formation, Inhibition, and Depletions from PBL. GPE were treated with neuraminidase to enhance rosette formation; GE were used untreated (9). Rosettes were obtained by mixing 0.1 ml of a 1% suspension of GP or G E with 0.1 ml of PBL at 2×10^6/ml in RPMI-1640-FCS. After incubation for 10 min in a 37°C water bath, the tubes were centrifuged for 5 min at 200*g* and then placed on ice for 2 hr. The cells were gently resuspended and 300 cells were scored for rosettes; a gentian violet stain was used to facilitate identification of a nucleated cell within each rosette. Inhibition of rosette formation was accomplished by incubating PBL with the desired antibody for 30 min in the cold. The cells were then washed and reacted with either G or GP E for rosette formation. Depletion of GE- or GPE-rosetting cells from PBL was accomplished by mixing equal volumes of a 1% suspension of E and PBL (3×10^6/ml) and incubating the cells at 37°C for 10 min. The cell suspension was then centrifuged for 10 min at 200*g* and was placed on ice for 2 hr. The cell pellets were resuspended and the cells were layered on a Ficoll-Hypaque gradient. The nonrosetting band of cells was recovered and, after washing, the cells were resuspended in RPMI-1640-FCS to the desired concentration.

Immune-Rosette Depletion of Antibody-Treated PBL. Sheep RBC were conjugated with affinity-purified goat anti-mouse IgG (G-AMIgG) antibody following treatment of the RBC with $CrCl_3$. In brief, 0.6 ml of a 0.6% solution of $CrCl_3$ was slowly added to a 0.5-ml suspension containing 0.1 ml of packed sheep RBC and 0.1 mg of G-AMIgG. The mixture was incubated for 1 hr at 30°C, washed with RPMI-1640, and the RBC concentration was adjusted to 5% in RPMI-1640-FCS. One milliliter of the IgG-coated sheep RBC suspension was added to 0.5 ml of PBL (20×10^6/ml) that had been treated with the monoclonal antibodies to be tested; the cell suspension was pelleted by centrifugation and was incubated for 1 hr at 4°C. The cells were gently resuspended and layered on a Ficoll-Hypaque gradient, and the nonrosetting band of cells were collected after centrifugation. After washing with iHBSS, the recovered PBL were resuspended to the desired concentration in RPMI-1640-FCS.

Reverse Hemolytic Plaque Assay. Stimulation of cat PBL for polyclonal induction of Ig synthesis by PWM was performed as described previously (11). In brief, 1 μg of PWM in a 50-μl volume was added to 1 ml PBL at $0.75–1.5 \times 10^6$/ml. After cultivation for 7 days in a humidified 10% $C0_2$ incubator at 37°C, the cells were recovered and plaque analysis was performed with Protein-A coated sheep RBC. Plaque forming cells (PFC) were visualized in agarose on glass slides, using a rabbit anti-cat IgG antiserum and GP complement at predetermined optimal concentrations.

IL-2 Production and Assay. IL-2 production and assay were performed as previously reported (10). In brief, PBL were incubated overnight in tissue culture flasks for 24 hr in a humidified 10% CO_2 incubator at 37°C and the following day, 1×10^6 cells in flat-bottom tissue culture wells were stimulated with 20 μg of ConA. The cells were cultured overnight in the incubator and supernatants were collected under sterile conditions for assay of IL-2 by the method of Gillis *et al.* (12) using the IL-2-dependent murine HT-2 cell line. Assays for Il-2 production were made in duplicate with variation of 10%–15% between samples.

Lectin Isolation of T Lymphocytes. Purified T lymphocytes from PBL were obtained by affinity chromatography using a *Pisum sativum* agglutinin (PSA)-linked Sepharose 6MB column. As reported previously (13), the higher affinity binding of PSA to B lymphocytes permitted depletion of B lymphocytes from PBL. leading to a pure population of feline T lymphocytes as defined by rosetting with G and GP E.

Monoclonal Antibodies. Balb/c mice were immunized with thymocytes from kittens and the splenocytes were fused to SP2/0 cells with polyethylene glycol following the procedure of Kennett *et al.* (14). Hybridoma supernatants were screened with PBL and thymocytes with a FACScan analyzer. After preliminary functional assays, selected hybridomas were subcloned for monoclonal antibody development.

FACScan Analysis. Cells (0.025 ml; 5×10^5 cells) were incubated with 0.025 ml of hybridoma supernatant culture fluids in the cold for 40 min. The cells were washed three times with phosphate-buffered saline (PBS) contain-

ing 0.1% NaN_3 and 0.5% bovine serum albumin and to the pellets was added 0.025 ml of a goat anti-mouse IgG/IgM antiserum conjugated with fluorescein isothiocyanate. The cells were incubated in the cold for 40 min, and after washing, were fixed with paraformaldehyde, and the percentage of stained cells was determined with a FACScan analyzer (Becton-Dickinson, Mountain View, CA).

Immunoprecipitation and SDS-PAGE Analysis of Membrane Antigens. Five million feline PBL were washed twice with PBS, surface labeled with 1 mCi $Na^{125}I$ (ICN, Costa Mesa, CA) in the presence of Iodogen (Pierce, Rockford, IL), and antigens were immunoprecipitated with monoclonal antibodies as described (15) except for the following modifications. Solubilization buffer (4°C) consisted of 1% Triton, 20 mM Tris-HCl (pH 7.8), 0.14 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% NaN_3, and 5 mM NaI. Solubilized membranes were centrifuged (16,000*g* for 15 min), and were precleared by rocking for 1 hr at 4°C with 1/10 vol glycine-quenched cyanogen bromide-activated agarose beads (Sigma, St. Louis, MO) followed by centrifugation (2,000*g* for 2 min). Aliquots of supernatant (250 μl) were diluted with 150 μl of Solubilization buffer and were then immunoprecipitated by rocking (4°C for 1 hr) with 100 μl monoclonal antibody tissue culture supernatant or control RPMI-1640-FCS medium containing 60 μg/ml normal mouse IgG followed by rocking (4°C for 1 hr) with 60 μl of 1/3 suspension of agarose bead-coupled goat anti-mouse Ig. Beads were washed as described, and immunoprecipitated proteins were solubilized by heating (100°C for 3 min) in 3% SDS nonreducing sample buffer followed by centrifugation (16,000*g* for 2 min), dividing each supernatant, and adjustment of one portion to 100 mM dithiothreitol-reducing agent. Unreduced and reduced supernatants were analyzed on SDS-10% PAGE Laemmli slab gels (16) with prestained Low-Range SDS-PAGE standards (Bio-Rad, Hercules, CA). Gels were dried, exposed to MR film (Kodak, Rochester, NY) with intensifying screens, and developed.

Results

Direct Activity of GE- and GPE-Rosetting Cells. Our previous data suggesting that GE- and GPE-rosetting cells help to express helper and suppressor functions, respectively, were obtained through removal of the cells from the reaction assay, i.e., an evaluation of the effect of their absence in a PWM-driven system for Ig production in the reverse hemolytic plaque assay. Removal of GPE-rosetting cells from PBL led to a marked decrease in the number of Ig-producing PFC, whereas removal of the GE-rosetting cells yielded a PFC response 2- to 3-fold above the normal control (9, 10). To substantiate the function of these cells in a direct manner, we developed the experimental schema shown in Figure 1 and Table I. We had reported that feline T lymphocytes could be isolated from PBL by affinity chromatography using the plant lectin, PSA (13). Thus, the high binding affinity of B lymphocytes to PSA made it possible to deplete PBL of these cells when passed through

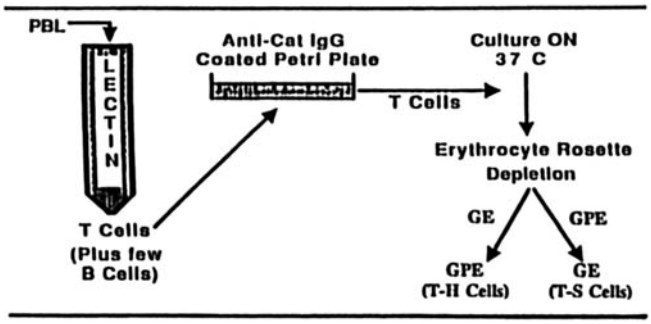

Figure 1. Schema showing the procurement of T lymphocytes from PBL by affinity chromatography using a PSA-linked Sepharose 6MB column (13). The few remaining B cells are removed from this preparation by adsorption on petri plates coated with a rabbit anti-cat IgG antiserum. The T cells are cultured overnight at 37°C, and rosette depletions with gerbil (GE) and guinea pig (GPE) erythrocytes are performed on aliquots to yield cells with receptors for GPE (T-H) and GE (T-S), respectively.

a PSA-linked Sepharose-6MB column, yielding a pure population of T lymphocytes. As shown in Figure 1, these cells were next placed on a petri plate coated with a rabbit anti-cat IgG antibody to remove any B lymphocytes that may have come off the column. The recovered cells were then cultured overnight at 37°C in a CO_2 incubator because this results in the maximum percentage of GPE-rosetting cells having helper activity (10). Aliquots of the cells were next depleted of GE- and GPE-rosetting cells, yielding, respectively, a population of cells having the GPE-receptor and a second population having the GE-receptor (Fig. 1; the T-H and T-S designations given to the two rosetting populations are used to denote functional activities previously reported for these cells in the assay used).

The cells obtained through the schema outlined in Figure 1 were tested for their ability to alter the function of target cells in a PWM system for Ig production as measured by PFC. The control target cells were fresh cells from the same donors in which depletions of GE- and GPE-rosetting cells had been made; the former would be capable of synthesizing Ig, whereas the latter cells would not (9). The results of three experiments are given in Table I. Note that the control GE-rosette depleted populations, i.e., no T cells added from schema of Figure 1, show a significant PFC response (486, 328, and 525 PFC), whereas only background PFC activity (30, 13, and 29 PFC) was obtained with the control target GPE-rosette depleted cells. In each experiment, the addition of the GE (T-S) cells from the schema of Figure 1 to the control GE-rosette depleted populations lowered the PFC response by more than 60%, whereas the addition of the GPE (T-H) cells from the schema of Figure 1 to the GPE-rosette depleted control cells increased the number of PFC 10- to 15-fold. Additional controls in this study show the lack of a PFC response when the GPE (T-H) cells and the GE (T-S) cells from schema of Figure 1 were cultured alone (Table I), indicating the absence of any B lymphocytes in these populations.

Monoclonal Antibodies. Supernatants from hybridoma cultures resulting from the fusion of SP2/0 my-

Table I. PFC Response as Affected by GE- and GPE-Rosetted Cells[a]

Cells added	Experiment 1				Experiment 2				Experiment 3			
	GE[b]	GPE[b]	GPE[c] (T-H)	GE[c] (T-S)	GE	GPE	GPE (T-H)	GE (T-S)	GE	GPE	GPE (T-H)	GE (T-S)
None	486[d]	30	32	30	328	13	9	12	525	29	23	10
GE[c] (T-S)	202	—[e]	—	—	105	—	—	—	176	—	—	—
GPE[c] (T-H)	—	274	—	—	—	228	—	—	—	311	—	—

[a] See Figure 1 schema.
[b] Control target cells: GE, gerbil erythrocyte-depleted population; GPE, guinea pig erythrocyte-depleted population.
[c] E-rosetted cells from lectin isolated T cells (Fig. 1).
[d] Values represent number of PFC in cultures stimulated with PWM. Number of cells in each culture: 0.75×10^6 for GE, GPE, GE (T-S), and GPE (T-H).
[e] Not done.

eloma cells and splenocytes from mice immunized with cat thymocytes were screened by FACScan analysis with PBL and thymus cell targets. Several were selected for further study based on the percentage of cells stained with these tissues. To verify the presumptive T cell specificity of the antibodies, two functional assays were used, the plaque assay for PWM-induced Ig synthesis and the production of IL-2 by PBL following ConA stimulation. PBL were treated with the antibodies and the antibody-bound cells were removed from the suspension by immune-rosetting with sheep RBC coated with goat anti-mouse IgG antibody ("Materials and Methods"). As shown in Table II with the hemolytic plaque assay, removal of cells reactive with antibody CT87 yielded a marked increase in the number of PFC relative to the nontreated control cells; in contrast, treatment of the same PBL suspensions with antibodies CT248 and CT843 led to a decrease in Ig synthesis as indicated by the 3-to 15-fold reduction in PFC. Table III shows comparable data for these antibodies in the IL-2 assay, i.e., an increase in IL-2 synthesis following depletion of CT87-reactive cells from PBL and a decrease in IL-2 production with removal of CT248- and CT843-treated cells.

The data in Tables II and III suggest that CT87 was reactive with cells capable of exerting a suppressive effect and antibodies CT248 and CT843 with cells contributing positively in the assay systems. Although the latter two antibodies appeared to be functionally equivalent in the two

Table II. PFC Response of Cat Peripheral Blood Leukocytes Following Removal of Cells Reactive with Antibodies CT87, CT248, and CT843[a]

PBL treatment	Experiment 1	Experiment 2	Experiment 3
None	251[b]	370	479
CT87	539	620	785
CD248	92	75	22
CD843	90	50	30

[a] PBL were treated with antibodies and, after washing, were mixed with sheep RBC coated with G-AMIgG for immune-rosette depletions ("Materials and Methods").
[b] Numbers represent PFC per 1×10^6 cells stimulated with PWM. Values are the mean of triplicate cultures with maximum variation of 15% PFC between cultures.

Table III. IL-2 Production by Cat Peripheral Blood Leukocytes Following Removal of Cells Reactive with Antibodies CT87, CT248, and CT843[a]

PBL treatment	Experiment 1	Experiment 2	Experiment 3
None	36[b]	8	21
CT87	52	23	42
CT248	22	3	7
CT843	10	2	3

[a] PBL were treated with antibodies and, after washing, were mixed with sheep RBC coated with G-AMIgG for immune-rosette depletions.
[b] IL-2 units/ml determined as described in "Materials and Methods."

assays, FACScan analyses of PBL revealed a difference in cell specificity. As shown in Figure 2 with tests on PBL from three animals, the percentage of cells stained with CT843 was markedly greater than that found with CT248; indeed, the percentage of cells reactive with CT843 approximated the total number of cells stained with CT248 and CT87. With PBL from 10 animals, the percentage cells stained with CT843 was 66.5 (SE ± 4.3; range 39–85); the percent reactive with CT248 was 42.2 (SE ± 2.4; range 30–53); and the percentage stained with CT87 was 26.9 (SE ± 3.5; range 14–44).

Immunoprecipitation and SDS-PAGE Analysis of Antigens Recognized by Monoclonal Antibodies CT843, CT87, and CT248. The functional and quantitative data presented above would suggest that antibody CT843 was reactive with all T lymphocytes in PBL, and antibodies CT87 and CT248 were identifying the CD8 and CD4 T-cell subsets, respectively. The selected hybridomas were subcloned and the resulting monoclonal antibodies were analyzed for their reactivity to surface membrane proteins on blood lymphocytes. PBL were surface-labeled with ^{125}I using Iodogen, and the membrane antigens were solubilized with buffer containing Triton X-100. Immunoprecipitation was performed with each monoclonal antibody or control mouse IgG, and the radiolabeled precipitates were analyzed under nonreducing and reducing conditions by SDS-PAGE and autoradiography. Figure 3 reveals that CT248 immunoprecipitates an antigen that migrates at M_r ~60 kDa under both nonreducing and reducing conditions.

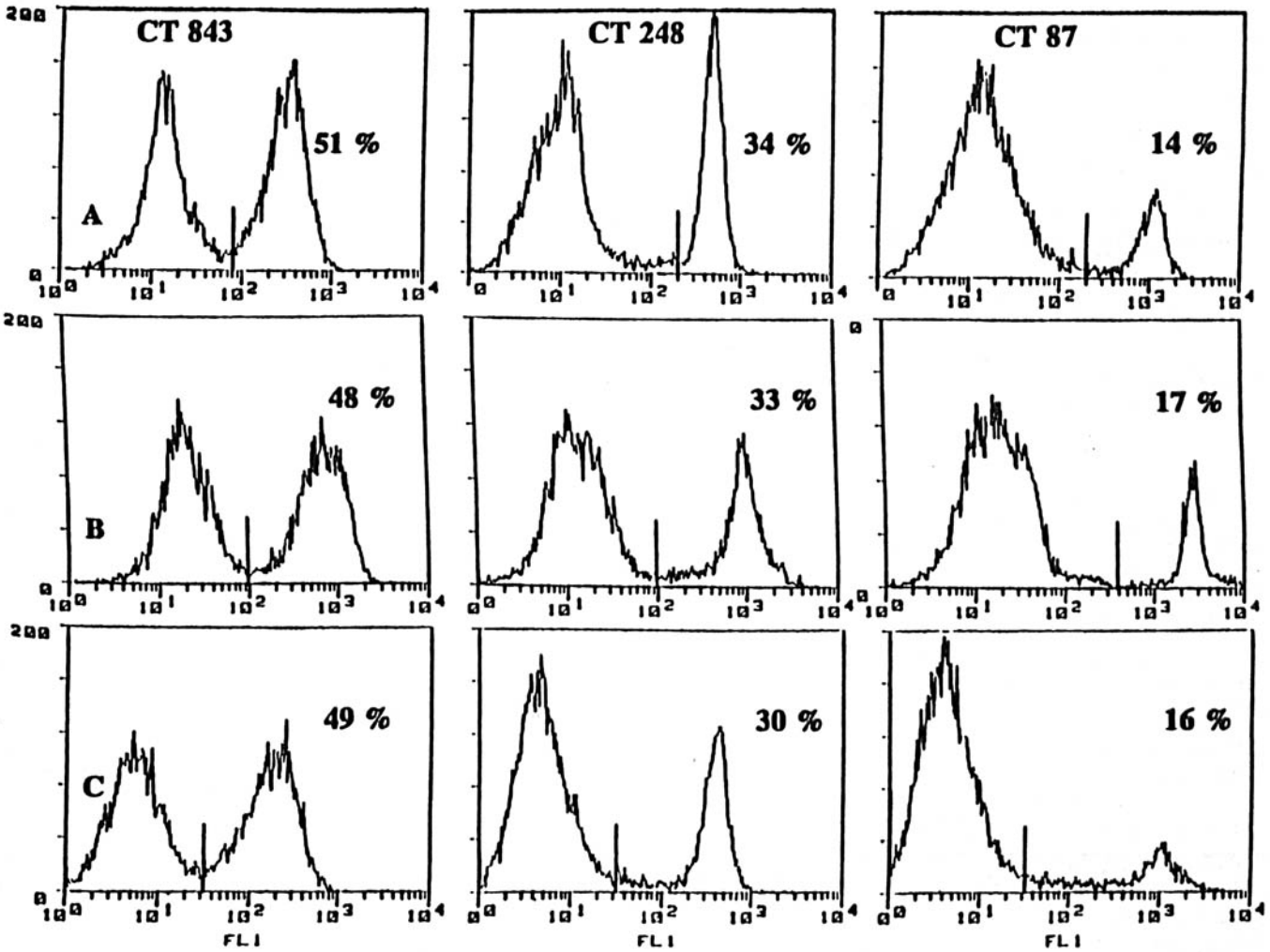

Figure 2. FACScan histograms of PBL from three cats (A–C) following immunofluorescent staining with CT843, CT248, and CT87.

Under reducing conditions, CT87 immunoprecipitated one band with an M_r of ~35 kDa; the high background of the control lane precludes clear resolution of a second band at ~39 kDa. Under nonreducing conditions, the band was not well visualized, possibly because it was not appreciably released from the antigen-antibody-agarose bead complex. Monoclonal antibody CT843 immunoprecipitated a heterodimer of M_r ~70 and ~60 kDa under both reducing and nonreducing conditions.

Rosettes and Monoclonal Antibody Correlates. We had suggested in an earlier study that monoclonal antibody CT87 was specific for CD8 cells based on inhibition of GE- but not GPE-rosette formation after treatment of PBL with this antibody (9). Rosette inhibition tests were performed with monoclonal antibodies CT248 and GPE because the latter was found to bind to cells expressing a helper function. As shown in Table IV, whereas pretreatment of PBL with monoclonal antibody CT87 specifically inhibited GE rosette formation, monoclonal antibody CT248 did not alter rosette development with either GE or GPE. Not shown in this table are similar rosette inhibition studies with monoclonal antibody CT843, the presumptive pan-T antibody; neither GE- nor GPE-rosette formation was blocked by this antibody. We had reported previously that PBL cultured overnight have shown a significant increase in the percentage of GPE-rosetting cells, and the *in vitro* tests have indicated these cells also to express a helper function. Aliquots of PBL from the same animals were cultured overnight and the cells were treated with monoclonal antibody CT248 for rosette inhibition; the percentage of GPE- and GE-rosetting cells did not differ from the controls (data not shown in Table IV).

While an overnight culture of PBL yields an increased percentage of GPE-rosettes, there is little or no change in GE-rosetting cells. Therefore, FACScan analyses were performed with monoclonal antibodies CT248 and CT87 on cultured PBL to determine whether there was any correlation with the percentage of cells stained and those rosetting with GP and G E, respectively. As shown in Table V, the percentage of GE-rosetting cells showed an excellent correlation to the percentage cells stained with monoclonal antibody CT87; the correlation between GPE-rosetting cells and staining with monoclonal antibody CT248, however, was not consistent, e.g. tests on PBL from two animals, nos.

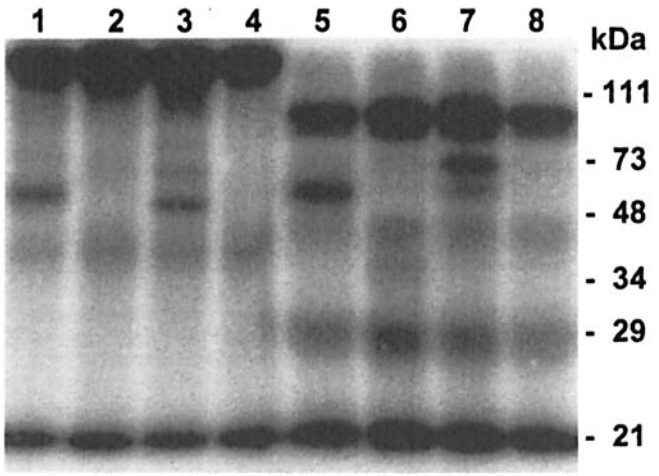

Figure 3. SDS-10% PAGE analysis of ^{125}I-labeled feline PBL immunoprecipitated with mAbs. Feline PBL were labeled with ^{125}I, solubilized, and immunoprecipitated with mAbs or control normal mouse IgG as described in "Materials and Methods." Immunoprecipitated proteins were solubilized with 3% SDS sample buffer and analyzed under nonreducing (lanes 1–4) and reducing (lanes 5–8) conditions on SDS-10% PAGE slabs that were then dried and autoradiographed. Lanes are as follows: 1 and 5, mAb CT248; 2 and 6, mAb CT87; 3 and 7, mAb CT843; 4 and 8, normal mouse IgG (control). Molecular weight markers in kilodaltons are indicated along the right margin.

Table IV. Rosette Formation of Blood Leukocytes with GE is Inhibited by mAb CT87, Whereas Rosette Formation with GPE Is Not Inhibited by mAbs CT87 and CT248

Animal number	Control		CT87 treated[a]		CT248 treated[a]	
	G	GP	G	GP	G	GP
	Percentage of rosettes					
1	22	16	0	21	21	18
2	10	22	0	20	10	24
3	25	32	2	34	22	30
4	33	25	2	20	30	22
5	28	17	1	15	26	17

[a] Blood leukocytes were incubated with the mAb for 30 min in the cold and, after washing, the cells were reacted with either G or GPE for rosette development as described in "Materials and Methods."

1 and 6, showed a marked quantitative difference for these two markers; 27% vs 47% for no. 1 and 30% vs 45% for no. 2. The differences were verified in repeat tests.

Two Populations of GPE-Rosetting and CD4 Cells in the Cat. Monoclonal antibody CT248, having an apparent specificity for the CD4 subset, permitted an examination of the relationship of this antibody to the two GPE-rosetting populations, i.e., those present on fresh PBL and those appearing after an overnight incubation of cells at 37°C, each shown previously to contribute to the production of Ig and IL-2 by PBL following stimulation with the lectins. GPE rosette depletions were performed on PBL on Day 0, i.e., the day of collection, and on another aliquot of the same PBL that had been cultured overnight, i.e., Day 1. The GPE rosette-depleted population on each day was stained with monoclonal antibody CT248. As shown in Table VI, removal of GPE-rosetting cells from Day 0 cells led to only a 17%–37% decrease in the percentage of cells stained with monoclonal antibody CT248; in contrast, GPE-rosette depletions of the Day 1 PBL, which showed the anticipated marked increase of GPE-rosetting cells, led to a significantly greater loss of cells reactive with CT248, this ranging from 75%–92% among the five PBL samples tested.

Table V. Percentage of PBL Reactive with mAbs CT87 and CT248 by FACScan Analysis Compared with Percentage of PBL rosetting with G and GPE[a]

Animal number	FACScan analysis		Erythrocyte rosetting	
	CT87	CT248	G	GP
	Percentage			
1	22	27	25	47
2	14	41	14	42
3	7	39	8	34
4	9	27	10	22
5	26	23	28	26
6	24	30	22	45

[a] PBL had been incubated overnight at 37°C before tests.

Discussion

Our original observation on the dichotomy of feline T cells as revealed through their E-rosetting properties represents a rather unique finding among mammalian species. Prior to the development of monoclonal antibodies, GPE rosettes were used as a pan-T cell marker (6–8), but the large range in percentages reported for feline lymphocytes rosetting with GPE suggested the latter cells were not optimal for this test, prompting a screening in our laboratory with E from several species. Thus, the finding of an apparent T lymphocyte distinction demonstrated with GPE-and GE-rosetting was serendipitous. The helper and suppressor

Table VI. Effect of GPE-Rosette Depletion on Cells Reactive with mAb CT248

PBL[a]	Control		Rosette depleted	
	GPE rosettes	MAb CT248	GPE rosettes	mAb CT248
	Percentage			
Day 0	28	35	<1	29
	11	21	<1	16
	10	35	<1	24
	15	28	<1	18
	22	27	<1	17
Day 1	42	41	<1	6
	26	23	1	2
	28	41	1	6
	27	35	2	9
	45	30	<1	5

[a] Day 0 indicates PBL were used on day of procurement, and Day 1 indicates PBL were cultured overnight at 37°C in incubator before testing.

functions attributed to those cells rosetting with GPE and GE, respectively, were based on quantitative alterations, positive or negative, in functional assays resulting from the removal of the rosetting population from the reaction system, i.e., an indirect test. One of the objectives of the present study was to demonstrate the activity of the E-rosetted cells by their addition to a reaction milieu, in this instance, the PWM-Ig-induced assay through which the helper and suppressor functions were originally identified. The positive and negative effects shown in Table I by the GPE- and GE-rosetting cells developed from the schema of Figure 1 afford strong support to our initial study assigning their respective helper and suppressor activities.

The T lymphocyte specificities of monoclonal antibodies CT248, CT87, and CT843 may be derived from both the functional assays and biochemical data. The interdependent relationship of CD4 and CD8 cells in the lectin polyclonal activation of B and T lymphocytes has been described for the feline and human (1, 17–19), and the experiments presented in Tables II and III support the CD4 and CD8 designations for CT248 and CT87, respectively. The percentage distribution of the lymphocyte subsets and the ratio of cells stained with CT248 and CT87, 1.75 (SE ± 0.18; range 1.11–2.92) for 10 PBL preparations, correlate with that noted by others for the feline homologs of CD4 and CD8 and is similar to that reported for man (3, 20, 21). The pan-T classification of CT843 has been based in part on FACScan data showing this antibody to recognize the sum total of cells stained by CT248 and CT87. The average value of 66% lymphocytes stained with this antibody, ranging from 39% to 85% among the 10 animals tested, parallels data reported by Tompkins *et al.* (3) and Dean *et al.* (20) for this species. Although not shown in the text of the present study, CT843 has stained 94%–100% of thymus cells from five kittens (8–10 weeks old), whereas CT87 and CT248 have stained 70%–85% of cells from this tissue.

Biochemical analyses of the antigens recognized by antibodies reactive with CD4 molecules or their homologs in the feline, man, and other species precipitate an antigen of 55–59 kDa (3, 22–25); this correlates with the protein of ~60 kDa precipitated by monoclonal antibody CT248, which along with the functional data suggest that CT248 is identifying the feline CD4 homolog. The CD8 homolog of man and that reported for the feline is a molecule of ~66–71 kDa, composed of two subunits ranging from ~31 to ~38 kDa under reducing conditions (1, 3, 24, 25). In this study, molecular analysis of the antigen recognized by monoclonal antibody CT87 under reducing conditions revealed one subunit at ~35 kDa; although a second band could not be clearly distinguished, the functional studies in Tables II and III suggest CT87 is also identifying the feline CD8 homolog. Of interest is the antigen recognized by monoclonal antibody CT843, which we have designated as a pan-T antibody. The heterodimer of ~70 and ~60 kDa immunoprecipitated by CT843 under both reducing and nonreducing conditions is to be contrasted to the protein recognized by the feline pan-T monoclonal antibody reported by Tompkins *et al.* (3). The latter, noted as antibody 1.572, precipitated a large molecule of ~120 kDa under both reducing and nonreducing conditions. Antibodies reactive with pan-T cell molecules of the human and other vertebrates may be found among the CD2, CD3, and CD5 homologs. Molecular weight characterization of the polypeptides recognized by the respective antibodies have ranged from 22 to 28 kDa for the molecular complexes of CD3, 45 to 58 kDa for CD2, and 67 to 69 kDa for CD5 (24–27).

The inhibition of GE-rosette formation by monoclonal antibody CT87 was reported in our initial study describing the T lymphocyte specificities of GE- and GPE-rosetting cells. Analogous to the human system wherein sheep RBC rosette formation with T lymphocytes is blocked by antibody to the CD2 complex (26–28), the CT87 monoclonal antibody reacts either with the GE receptor itself or with a closely associated structure; the absence of rosette inhibition with monoclonal antibodies CT248 and CT843 indicates that the receptors for E (GP) rosetting are different from the antigenic site(s) to which the antibodies are binding. Rosette formation with GE also appears to be restricted to lymphocytes, an observation underscored by the excellent correlation in the percentage of cells stained with monoclonal antibody CT87 and the percentage of GE-rosetting cells. The lymphocyte specificity of GE, however, contrasts to that of GPE where we and others have observed that a small percentage of monocytes and granulocytes, when present in PBL preparations, will also rosette with the latter cells (5, 9, 29); indeed, bone marrow cells have also shown a high percentage of rosetting with GPE (9). This lack of cellular specificity and variable expression of the GPE receptor on lymphocytes (see below) may account for the differences noted in the FACScan data with CT248 and the percentage of cells rosetting with GPE.

In a previous study, we had documented the presence of two populations of GPE-rosetting cells, those present on fresh Day 0 PBL, and those appearing after overnight culture of the cells at 37°C, i.e., on Day 1 PBL. Both populations of GPE-rosetting cells were shown to have a T helper function in the two *in vitro* assays used (10). The consistency of the appearance of the second GPE-rosetting population and its helper function prompted the suggestion that we were recognizing two subsets of CD4 cells, one having the GPE receptor on fresh cells and the second showing the development of this receptor on another lymphocyte population after overnight culture. That the latter was a manifestation of a metabolic process was revealed by inhibition of the appearance of the GPE receptor on this second population when the cells were cultured overnight in the presence of cycloheximide, a protein synthesis inhibitor (10). The availability of monoclonal antibody CT248, having an apparent specificity for CD4 cells, permitted us to test the thesis that the GPE-rosetting procedure was identifying two subsets of T helper cells. It was speculated that the depletion of the GPE-rosetting cells from fresh PBL on Day 0 should

not remove all cells reactive with CT248, the CD4 monoclonal antibody. Conversely, GPE-rosette depletions performed on cells cultured overnight, when both populations of GPE-rosetting cells were present, should remove all cells reactive with monoclonal antibody CT248. The results of such an experiment have supported this thesis: GPE rosette depletions of five PBL preparations on Day 0 cells left behind 63%–83% of cells still reactive with CT248, whereas a similar depletion of the Day 1 PBL, those cultured overnight, showed only 8%–25% of the remaining cells to be reactive with CT248 (Table VI). That the latter depletion did not remove all CT248-reactive cells may reflect technically incomplete rosette depletion or the presence of CD4 cells on which the GPE receptor had not yet developed. The existence of such cells was revealed in an earlier study when it was observed that following GPE-rosette depletions of Day 1 PBL cultivation of the remaining cells for 6 days resulted in the appearance of an additional small percentage (6%–8%) of GPE-rosetting cells, indicating a slow or delayed expression of this receptor on a few lymphocytes (10). The present study with monoclonal antibody CT248 and GPE rosette depletions supports our original suggestion of two subsets of CD4 cells identified by the GPE-rosetting phenomenon. The biologic and immunologic significance of lymphocytes acquiring an E-rosetting receptor after *in vitro* culture is yet to be determined; i.e., functional differences not readily apparent may exist between the two subsets of the CD4 cells.

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