resuspended in the rosetting medium to 5% cell concentration, stored at 4°C and used within 5 days. Before use, 1 ml of 5% SRBC stock was washed with 10 ml of rosetting medium and resuspended in 5 ml of rosetting medium to obtain 1% cell concentration.

Treatment of SRBC with dextran. SRBC were treated with dextran by a modification of the procedure described by Wardley (1977). Briefly, 200 μl of washed, packed SRBC were mixed with 6% w/v dextran (Dextran $T_{.70}$ Pharmacia Fine Chemicals, Uppsala, Sweden) in physiological saline to obtain 1% cell concentration for the rosette assay.

Rosette assay. Lymphocytes (100 μl) resuspended in the rosetting medium at 10^7 cells/ml were added to 200 μl of 1% SRBC suspension in small conical 1 ml centrifuge tubes (Analytic Lab Accessories, West Hempstead, N.Y) and incubated in 37°C water bath for 10 min. The cells were then centrifuged at 200 *g* for 5 min at room temperature. After overnight incubation at 4°C, a drop of 0·5% trypan blue was added, and the cells were resuspended by gently rocking the tube lightly until the cells looked homogenously dispersed. A drop of the suspension was placed in a haemocytometer and 200 live cells were counted. A lymphocyte with three or more adherent erythrocytes was termed a rosette-forming cell (RFC). Each sample was set up in duplicate and results were expressed as mean of two determinations.

Quantitation of Ig-bearing cells. Surface membrane immunoglobulin (sIg) bearing cells were identified by a previously described method (Paul *et al.*, 1977b). Briefly, 1×10^6 cells were added to small conical centrifuge tubes and washed three times in PBS containing 0·2% sodium azide and 1% bovine serum albumin, and pelleted by centrifugation at 800 *g* for 2 min. The pellets were resuspended in 50 μl of 1:10 dilution of fluoresceinated rabbit anti-bovine IgG (heavy and light chains, Lot S402, Miles Laboratories, Elkhart, Indiana), vortex mixed and incubated at 4°C for 30 min with intermittent mixing. After incubation, the cells were washed three times with PBS containing sodium azide and BSA, and examined by alternating phase and fluorescent microscopy. The percentage of cells positive for sIg was determined by counting 200 cells.

Separation of lymphocyte populations on nylon-wool columns. Lymphocytes were separated on nylon-wool columns into B-cell-enriched nylon-adherent and T-cell-enriched non-adherent populations (Paul *et al.*, 1977b). Columns were prepared in 12-ml syringes packed with 0·6 *g* nylon wool (Leuko-Pak; Fenwal Laboratories, Morton Grove, Illinois) to the 6-ml mark, sterilized, and equilibrated at 37°C with HBSS containing 5% FCS. PBL (150×10^6) were applied to each column and incubated at 37°C for 30 min. The columns were slowly washed at a flow rate of 1 ml/min with HBSS containing 5% FCS and the cells eluted in the first 15 ml were pooled and referred to as non-adherent cells. The columns were then washed with 200 ml of HBSS containing 5% FCS pre-warmed at 37°C. The adherent cells were harvested by adding HBSS to the column and compressing the nylon wool with the plunger to exude the cells. This process was repeated and the adherent cells were pooled.

RESULTS

Development of a rosette assay for quantitation of bovine T lymphocytes

The following experiments on rosette formation were designed to study the enhancing effect of AET and dextran treatment of SRBC. As shown in Table 1, untreated SRBC resuspended in the rosetting medium (RPMI 1640 containing 10% FCS) formed a small percentage of rosettes (5·8 ± 3·1%) with PBL from three normal animals. Both AET and dextran treatment enhanced the rosette formation. A mean of

TABLE 1. Effect of treatment of sheep erythrocytes with dextran and various concentrations of AET on rosette formation by normal bovine peripheral blood lymphocytes

Treatment of sheep erythrocytes	RFC (% ± s.d.)*
None	5·8 ± 3·1
6% Dextran	31·1 ± 12·4
0·1 M AET	59·3 ± 9·3
0·143 M AET	30·5 ± 22·3
0·2 M AET	1·7 ± 1·5

AET = 2-aminoethylisothiouronium bromide; RFC = rosette-forming cells with three or more adherent sheep erythrocytes.

* Percentage expressed as the mean value from three animals after counting a minimum of 200 cells from each sample.

In cattle, a satisfactory rosette assay is lacking. Untreated SRBC bind to a very small percentage of thymocytes in 100% FCS (Grewal, Rouse & Babiuk, 1976; Higgins & Stack, 1977). However, rosette formation is enhanced by neuraminidase treatment of SRBC with up to 50% of thymocytes and 38% peripheral blood lymphocytes (PBL) forming rosettes in 100% FCS (Grewal *et al.*, 1976; Higgins & Stack 1977). These percentages are lower than that reported in man and other animals (Jondal *et al.*, 1972; Johansen *et al.*, 1974). Recently, Wardley (1977) has reported that approximately 70% bovine PBL form rosettes with SRBC in the presence of 6% dextran. In our laboratory, we examined the effect of dextran on rosette formation and detected only 30% rosette-forming cells (RFC) in bovine peripheral blood (Paul *et al.*, unpublished observations). The present study was initiated to develop an improved assay for the detection of bovine T lymphocytes and additionally to develop procedures for the identification of bovine monocytes which have not been identified satisfactorily in cattle. Human peripheral blood mononuclear cell fractions isolated by Ficoll–Hypaque flotation contain a large percentage (44%) of monocytes (Zucker-Franklin, 1974). In man, these cells can be differentiated from lymphocytes by peroxidase (Preud' Homme & Flandrin, 1974) and non-specific esterase (Horwitz *et al.*, 1977) staining phagocytosis of inert particles and adherence to glass or plastic surfaces (Territo, Golde & Cline, 1976). Their identification and quantitation is essential for the accurate estimation of T and B cells.

Thus, the present investigation was designed to (1) study the effect of AET treatment of SRBC on rosette formation by bovine T lymphocytes (2) develop a marker for bovine monocytes and (3) to determine the frequency of T cells, B cells and monocytes in peripheral blood and tissues of normal and lymphocytotic cattle.

MATERIALS AND METHODS

Source of animals. Normal 1-2-year-old cattle of Jersey and Holstein breeds were selected at random from the dairy herd of the Animal Science Department, University of Minnesota. Cows with persistent lymphocytosis were obtained from a multiple-incidence leukaemia herd and housed at the University of Minnesota. Three bovine foetuses, approximately 5–8 months of age, were obtained from a local abattoir. Foetal ages were estimated from crown to rump measurements (Roberts, 1971). Spleens and thymuses were also obtained from two normal Hereford cattle, approximately 14 months of age.

Lymphocyte suspensions. Peripheral blood was obtained by jugular venipuncture and placed in tubes containing heparin (50 units/ml blood). Lymphocytes were separated by the Ficoll–Hypaque technique (Böyum, 1968). Briefly, heparinized blood was diluted 1:1 with sterile physiological saline, layered onto Ficoll–Hypaque (specific gravity 1·08) and centrifuged at 400 ***g*** at 20°C for 30 min. The interface was carefully aspirated and washed twice with Hanks's balanced salt solution (HBSS). The cell preparation contained approximately 95% mononuclear cells and will be referred to as peripheral blood lymphocytes (PBL). Approximately 95 to 98% PBL were judged viable by trypan blue dye exclusion.

Splenic lymphocytes and thymocyte suspensions were obtained by mincing the tissue by scissors after removing the extraneous connective tissue. HBSS was added to the petri dishes and cell suspensions were transferred to 50-ml conical tubes. After the larger particles had settled, the supernatant rich in cells was aspirated and layered on Ficoll–Hypaque gradients After centrifugation at 400 ***g*** for 20 min, the interface was collected, washed three times with HBSS and examined for T and B cells.

Marker for monocytes. Monocytes were labelled by latex ingestion. Cell preparations were adjusted approximately to 2×10^6 cells/ml in HBSS and 1 ml of 1:50 dilution of latex particles of 0·81 μ diameter (Difco, Detroit, Michigan) were added to 10 ml of cell suspension. The mixture was incubated at 37°C for 1 hr with continuous rotation (Multipurpose rotator, Scientific Industries Inc., Springfield, Massachusetts). After incubation, the cells were washed three times with 50 ml HBSS. These cells were then stained for SIg or assayed for rosette formation. Cells ingesting three or more latex particles were referred to as monocytes.

Rosetting medium. FCS (Lot 40551107, Flow Laboratories, Rockville, Maryland) was heat inactivated and absorbed with SRBC. One volume of FCS was mixed with 0·2 volumes of packed SRBC and incubated in 37°C water bath for 1 hr. FCS–SRBC mixture was further incubated at 4°C for 1 hr, centrifuged at 400 ***g*** for 20 min and FCS carefully aspirated. Hepes-buffered RPMI-1640 (Biolabs Inc., Chicago, Illinois) containing 10% absorbed FCS was used as a rosetting medium throughout the study.

AET treatment of sheep erythrocytes. SRBC preserved in Alsever's solution were purchased from Wilfer Laboratory, Stillwater, Maine, and were used within 2 weeks. AET (Sigma Chemical Co., Saint Louis, Missouri) was dissolved in distilled water and the solution was adjusted to pH 9·0 by addition of 5N NaOH and sterilized by filtration. Before treatment, SRBC were washed three times with phosphate buffer saline, pH 7·2 and treated with 0·1 M, 0·143 M or 0·2 M freshly prepared AET as described by Pellegrino *et al.* (1975). Briefly, 4 vol of AET solution were added to 1 vol of packed SRBC and incubated with intermittent mixing at 37°C for 10, 15, 20, 25 or 30 min. After incubation, cells were washed three times with cold (4°C) physiological saline followed by two washes with the rosetting medium. Untreated and AET treated SRBC were

resuspended in the rosetting medium to 5% cell concentration, stored at 4°C and used within 5 days. Before use, 1 ml of 5% SRBC stock was washed with 10 ml of rosetting medium and resuspended in 5 ml of rosetting medium to obtain 1% cell concentration.

Treatment of SRBC with dextran. SRBC were treated with dextran by a modification of the procedure described by Wardley (1977). Briefly, 200 μl of washed, packed SRBC were mixed with 6% w/v dextran (Dextran $T_{,70}$ Pharmacia Fine Chemicals, Uppsala, Sweden) in physiological saline to obtain 1% cell concentration for the rosette assay.

Rosette assay. Lymphocytes (100 μl) resuspended in the rosetting medium at 10^7 cells/ml were added to 200 μl of 1% SRBC suspension in small conical 1 ml centrifuge tubes (Analytic Lab Accessories, West Hempstead, N.Y) and incubated in 37°C water bath for 10 min. The cells were then centrifuged at 200 *g* for 5 min at room temperature. After overnight incubation at 4°C, a drop of 0·5% trypan blue was added, and the cells were resuspended by gently rocking the tube lightly until the cells looked homogenously dispersed. A drop of the suspension was placed in a haemocytometer and 200 live cells were counted. A lymphocyte with three or more adherent erythrocytes was termed a rosette-forming cell (RFC). Each sample was set up in duplicate and results were expressed as mean of two determinations.

Quantitation of Ig-bearing cells. Surface membrane immunoglobulin (sIg) bearing cells were identified by a previously described method (Paul *et al.*, 1977b). Briefly, 1×10^6 cells were added to small conical centrifuge tubes and washed three times in PBS containing 0·2% sodium azide and 1% bovine serum albumin, and pelleted by centrifugation at 800 *g* for 2 min. The pellets were resuspended in 50 μl of 1:10 dilution of fluoresceinated rabbit anti-bovine IgG (heavy and light chains, Lot S402, Miles Laboratories, Elkhart, Indiana), vortex mixed and incubated at 4°C for 30 min with intermittent mixing. After incubation, the cells were washed three times with PBS containing sodium azide and BSA, and examined by alternating phase and fluorescent microscopy. The percentage of cells positive for sIg was determined by counting 200 cells.

Separation of lymphocyte populations on nylon-wool columns. Lymphocytes were separated on nylon-wool columns into B-cell-enriched nylon-adherent and T-cell-enriched non-adherent populations (Paul *et al.*, 1977b). Columns were prepared in 12-ml syringes packed with 0·6 *g* nylon wool (Leuko-Pak; Fenwal Laboratories, Morton Grove, Illinois) to the 6-ml mark, sterilized, and equilibrated at 37°C with HBSS containing 5% FCS. PBL (150×10^6) were applied to each column and incubated at 37°C for 30 min. The columns were slowly washed at a flow rate of 1 ml/min with HBSS containing 5% FCS and the cells eluted in the first 15 ml were pooled and referred to as non-adherent cells. The columns were then washed with 200 ml of HBSS containing 5% FCS pre-warmed at 37°C. The adherent cells were harvested by adding HBSS to the column and compressing the nylon wool with the plunger to exude the cells. This process was repeated and the adherent cells were pooled.

RESULTS

Development of a rosette assay for quantitation of bovine T lymphocytes

The following experiments on rosette formation were designed to study the enhancing effect of AET and dextran treatment of SRBC. As shown in Table 1, untreated SRBC resuspended in the rosetting medium (RPMI 1640 containing 10% FCS) formed a small percentage of rosettes (5·8 ± 3·1%) with PBL from three normal animals. Both AET and dextran treatment enhanced the rosette formation. A mean of

TABLE 1. Effect of treatment of sheep erythrocytes with dextran and various concentrations of AET on rosette formation by normal bovine peripheral blood lymphocytes

Treatment of sheep erythrocytes	RFC (% ± s.d.)*
None	5·8 ± 3·1
6% Dextran	31·1 ± 12·4
0·1 M AET	59·3 ± 9·3
0·143 M AET	30·5 ± 22·3
0·2 M AET	1·7 ± 1·5

AET = 2-aminoethylisothiouronium bromide; RFC = rosette-forming cells with three or more adherent sheep erythrocytes.

* Percentage expressed as the mean value from three animals after counting a minimum of 200 cells from each sample.

TABLE 2. Effect of time of AET treatment of sheep erythrocytes on rosette formation by bovine peripheral blood lymphocytes

Time of AET treatment (min)	RFC (%± s.d.)*
10	47·8± 17·6
15	56·1± 11·7
20	61·2± 9·8
25	59·3± 11·4
30	53·2± 10·7

AET = 2-aminoethylisothiouronium bromide; RFC = rosette-forming cell with three or more adherent sheep erythrocytes.

*Percentage expressed as the mean value from three animals by counting a minimum of 200 cells.

59·3±9·3% PBL with 0·1 M AET, 30·5±22·3 with 0·143 M AET, and 31·1±12·4% with 6% dextran-treated SRBC formed rosettes. Only a low percentage (1·7±1·5%) of rosettes were detected with 0·2 M AET-treated SRBC. It is clear from these results that 0·1 M AET-treated SRBC formed the highest number of rosettes with bovine PBL. Furthermore, the majority of the rosettes were large, easily quantitable and consisted of lymphocytes with 5–20 bound SRBC. Only a small percentage of lymphocytes had 3–4 bound SRBC. Thus, SRBC treated with 0·1 M AET were used in subsequent experiments.

To further standardize the assay, optimum time of AET treatment was determined. SRBC were treated with 0·1 M AET for various lengths of time and tested in rosette assay. As shown in Table 2, SRBC treated with AET for 20 min formed the highest percentage of rosettes and was used throughout the study. AET-treated SRBC could be stored at 4°C and successfully used for 5 days without any significant loss in rosette formation. Washing of AET-treated SRBC before use was essential to remove haemolysed SRBC for the detection of the maximum number of RFC.

Kinetics of rosette formation by bovine PBL with AET-treated SRBC

PBL from three normal cattle were incubated with AET-treated SRBC at 37°C for 10 min followed by centrifugation at 200 *g* for 5 min. After centrifugation, the samples were further incubated at 4°C for

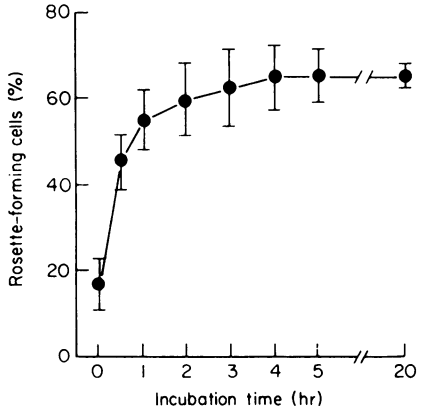

FIG. 1. Kinetics of rosette formation by bovine peripheral blood lymphocytes (PBL) with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes. Bovine PBL were incubated with AET-treated sheep erythrocytes at 37°C for 10 min, centrifuged at 200 *g* for 5 min and further incubated at 4°C for varying periods of time. Results are expressed as mean rosette-forming cells from three animals± s.d.

TABLE 3. Simultaneous detection of sIg-bearing and rosette-forming cells in unfractionated and nylon-wool column fractionated bovine peripheral blood lymphocytes

Animal No.	Lymphocyte Population	Percentage of cells			
		sIg^+ RFC^-	sIg^- RFC^+	sIg^- RFC^-	sIg^+ RFC^+
Normal					
	PBL	30·2	61·4	8·4	0
690	Nylon-adherent	74.0	15·5	10·5	0
	Non-adherent	1·0	90·0	9·0	0
	PBL	31·5	61·0	7·5	0
695	Nylon-adherent	57·8	40·4	1·8	0
	Non-adherent	1·9	78·6	19·5	0
	PBL	30·4	50·5	19·1	0
800	Nylon-adherent	57·5	30·5	12·0	0
	Adherent	2·0	77·0	21·0	0
Persistent lymphocytotic					
	PBL	77·7	21·9	0·5	0
39	Nylon-adherent	95·0	4·0	1·0	0
	Non-adherent	2·0	97·0	1·0	0
	PBL	60·5	34·5	5·0	0
214	Nylon-adherent	85·0	12·5	2·5	0
	Non-adherent	3·5	84·0	12·5	0

PBL = peripheral blood lymphocytes: sIg^+ = surface membrane immunoglobulin positive; sIg^- = surface membrane immunoglobulin negative; RFC^+ = positive for rosette formation with AET-treated sheep erythrocytes; RFC^- = negative for rosette formation with AET-treated sheep erythrocytes.

0, 1/2, 1,2,3,4,5 or 20 hr and examined for rosette formation. As depicted in Fig. 1, a small number of rosettes (17%) were detected immediately after centrifugation (0 hr). Rosette formation increased rapidly and reached maximum in 4 hr. The percentage of RFC detected did not change between 4 and 20 hr incubation time. Thus, for convenience, samples were incubated overnight for approximately 20 hr before counting, throughout the study.

Characterization of the cell population forming rosettes with AET-treated SRBC

The following experiment was designed to characterize the lymphocyte population forming rosettes with AET-treated SRBC. PBL from three normal and two lymphocytotic cattle were separated on nylon-wool columns into nylon-adherent and non-adherent cell populations. PBL, nylon-adherent and non-adherent cells were first stained for sIg and then processed for rosette formation. The cells containing one or both markers were identified.

As shown in Table 3, nylon-adherent cells from all animals were enriched in sIg-bearing cells and were depleted of RFC. In contrast, nylon-nonadherent cells were enriched in RFC but were depleted of sIg-bearing cells (Table 3). None of the lymphocytes with three or more adherent SRBC were positive for sIg. However, cells with one or two adherent SRBC often were positive for sIg. Consequently, cells with three or more adherent SRBC were counted as RFC. These experiments provided evidence that AET-treated SRBC form rosettes with nylon-nonadherent sIg negative, presumably T lymphocytes.

Distribution of sIg-bearing and rosette-forming cells in tissues of bovine foetuses and heifers

Thymocytes collected from three, 5–8 month-old foetuses formed an average of $86 \pm 3{\cdot}1\%$ (range 83–89%) rosettes with AET-treated SRBC, but did not contain sIg-bearing cells (Table 4). Thymocytes

TABLE 4. Distribution of sIg-bearing and rosette-forming cells in the thymus and spleen of bovine foetuses and heifers

No.	Approximate Age (months)	Thymus		Spleen	
		sIg$^+$ (%)	RFC (%)	sIg$^+$ (%)	RFC (%)
Foetuses*					
1	5	0	83·0	n.d.	n.d.
2	6	0	89·0	n.d.	n.d.
3	8	0	85·0	n.d.	n.d.
	Mean ± s.d.	0	85·7 ± 3.1		
Heifers					
1	14	0	89·8	17·5	43·8
2	14	0	82·6	7·5	70·0
	Mean ± s.d.	0	86·2 ± 5·1	12·5 ± 7·1	56·9 ± 18·9

sIg$^+$ = Surface membrane immunoglobulin bearing; RFC = rosette-forming cell with three or more adherent sheep erythrocytes; n.d. = not done.

*Gestational age of foetuses were estimated from crown-rump measurements (Roberts, 1971).

and splenic lymphocytes from two, 14-month-old heifers were also examined for sIg and rosette formation. Thymocytes contained 86·2 ± 5·1% (range 82·6–89·8%) RFC and 0% sIg-bearing cells, whereas splenic lymphocytes contained 56·9 ± 18·9% (range 43·8–70·0%) RFC and 12·5 ± 7·1% (range 7·5–17·4% sIg-bearing cells. Our data demonstrate that the majority of thymocytes form rosettes and lack sIg, thus providing additional evidence that AET-treated SRBC form rosettes with bovine T lymphocytes.

TABLE 5. Frequency of sIg-bearing and latex-ingesting cells in Ficoll–Hypaque separated peripheral blood lymphocytes from normal cattle

Animal No.	Percentage of cells*			
	sIg$^+$ Latex$^-$	sIg$^+$ Latex$^+$	Total sIg$^+$	Total Latex$^+$
1	7·0	8·6	15·6	18·0
2	4·4	4·0	8·4	29·6
3	12·1	5·6	17·7	23·2
4	7·4	3·3	10·7	12·0
5	8·9	2·8	11·7	10·5
6	17·3	9·2	26·5	25·7
7	8·4	7·6	16·0	19·8
8	11·8	5·3	17·1	16·8
9	13·7	4·7	18·4	16·4
10	7·9	6·6	14·5	28·5
11	18·5	2·8	21·3	10·1
Mean ± s.d.	10·7 ± 4.4	5·5 ± 2·3	16·2 ± 5·0	19·2 ± 6.9
Range	(4·4–18·5)	(2·8–9·2)	(8·4–26·5)	(10·1–29·6)

sIg$^+$ = Surface immunoglobulin positive; Latex$^+$ = cells ingesting three or more latex particles; Latex$^-$ = cells ingesting two or less latex particles.

*Minimum of 200 cells were counted.

TABLE 6. Frequency of sIg-bearing and rosette-forming cells in the peripheral blood of normal cattle

Animal number	Percentage of Lymphocytes*		
	sIg^+	RFC	sIg^- RFC^-
1	7·0	65·0	29·0
2	11·0	62·5	26·5
3	12·0	38·5	49·5
4	5·0	70·0	25·0
5	6·0	68·5	25·5
6	12·0	88·0	0
7	10·4	66·5	23·1
8	8·9	72·5	18·6
9	8·9	67·0	24·1
10	8·6	67·0	24·4
11	6·2	52·0	41·8
12	15·8	63·0	21·2
13	8·5	55·5	36·0
14	9·9	51·3	38·8
15	23·3	61·0	15·7
16	10·5	53·0	36·5
17	14·2	66·4	19·4
18	16·4	60·5	23·1
19	11·1	63·0	25·9
20	20·6	64·5	14·9
Mean ± s.d.	11·3 ± 4·75	62·8 ± 9·95	29·5 ± 10·85
Range	(5·0–23·3)	(38·5–88·0)	(0–49·5)

sIg^+ = Surface membrane immunoglubulin positive; sIg^- = surface membrane immunoglobulin negative; RFC = rosette-forming cell with three or more adherent sheep erythrocytes.

*Cells ingesting three or more latex particles were excluded. A minimum of 200 cells were counted.

Frequency and characterization of monocytes and B cells in bovine peripheral blood

To determine the frequency of monocytes and B cells in peripheral blood of cattle, PBL from eleven normal cattle were incubated with latex particles at 37°C for 1 hr, washed and then stained for the presence of sIg. Cells with three or more latex particles were referred to as monocytes. As shown in Table 5, there were 16·2 ± 5·0% (range 8·4–26·5%) sIg^+ cells. Only 10·7 ± 4·4% (range 8·4–26·5%) cells were latex negative, thus true B cells. There were 19·2 ± 6·9%) (range 10·1–29·6%) latex-ingesting cells in PBL preparations from normal cattle. These data demonstrate that bovine monocytes also bear sIg and these cells can be differentiated from B lymphocytes by latex phagocytosis.

Frequency of T and B lymphocytes in peripheral blood of normal and persistent lymphocytotic cattle

To determine the normal values of T and B lymphocytes in peripheral blood of cattle, PBL from twenty 1-2-year-old normal cattle were isolated and monocytes were identified by latex ingestion. T and B cells were quantitated by rosette formation with AET-treated SRBC and by the presence of sIg, respectively. Cells ingesting three or more latex particles were excluded from counting. Under these conditions, 62·8 ± 9·95% (range 38·5–88·0%) T and 11·3 ± 4·75% (range 5·0–23.3%) B cells were detected in PBL of normal cattle (Table 6). The percentage of null cells lacking both sIg and rosette formation was 25·9 ± 10·85% (range 0–49·5%) (Table 6).

TABLE 7. Frequency of sIg-bearing and rosette-forming cells in the peripheral blood of bovine leukaemia virus infected persistent lymphocytotic cows

Animal No.	Percentage of cells*		
	sIg^+	RFC	sIg^- RFC^-
1	70·0	20·0	10
2	49·4	42·0	8·6
3	57·0	42·0	1·0
Mean ± s.d.	58.8 ± 10·4	34·7 ± 12·7	6·5 ± 4·84

sIg^+ = Surface membrane immunoglobulin positive; sIg^- = surface membrane immunoglobulin negative; RFC = rosette-forming cells with three or more adherent sheep erythrocytes; RFC^- = cells with two or less adherent sheep erythrocytes.

*Cells ingesting three or more latex particles were not counted. Minimum of 200 cells were counted.

Several investigators have reported that the B-cell percentage is elevated in peripheral blood of cattle with persistent lymphocytosis, a prodromal stage of bovine leukaemia (Muscoplat *et al.*, 1974; Weiland & Straub, 1975; Paul *et al.*, 1977a; Kenyon & Piper, 1977; Kumar *et al.*, 1978). However, information on the frequency of T lymphocytes in peripheral blood of cattle with persistent lymphocytosis is lacking. Thus, with the development of improved methodology, we examined PBL from three persistent lymphocytotic cows for T and B cells. Again, cells ingesting three or more latex particles were excluded from counting. As expected, the B-cell percentage was increased in peripheral blood of these cows with a mean of 58·8 ± 10·4% (range 49·4–70·0%), and the T-cell percentage was decreased to 34·7 ± 12·7% (range 20–42%) (Table 7), thus confirming our earlier observation that bovine leukaemia is a B-cell disease resulting from over-production of B cells.

DISCUSSION

There has been a great deal of controversy regarding the methodology of rosette formation by bovine lymphocytes. Grewal *et al.* (1976) reported that 38% PBL and 52% foetal thymocytes form rosettes with neuraminidase-treated SRBC in the presence of 100% FCS. However, Higgins & Stack (1977) using this technique detected only 0% RFC in peripheral blood and 20% in foetal thymuses. These percentages are lower than the normal range of 50–80% RFC in peripheral blood and 80–90% in thymuses of man and other animals. (Jondal *et al.*, 1972; Johansen *et al.*, 1974). Recently, Wardley (1977) reported the enhancing effect of dextran on rosette formation by bovine PBL, similar to that observed by Brown *et al.*, (1975) in man. Wardley (1977) detected 70% RFC in bovine PBL and 96% in thymuses. These findings were partially substantiated in our laboratory, since 30% PBL formed rosettes with dextran-treated SRBC. This discrepancy might be due to the different source and molecular weight of dextran used in our studies.

In the present investigation, we have shown that SRBC treated with the sulfhydryl compound AET acquire a markedly enhanced ability to form rosettes with bovine PBL and offer an excellent tool for quantitation of T lymphocytes. This is evidenced by increased percentage of rosettes formed, increase in number of SRBC bound to lymphocytes and increased stability of these rosettes. The procedure described here is simple and AET-treated SRBC can be stored at 4°C and used for at least 5 days without loss in their ability to form rosettes. AET has also been used for the enhancement of rosette formation by human lymphocytes (Kaplan & Clark, 1974; Pellegrino *et al.*, 1975). The optimum conditions of SRBC

treatment with AET were different in the bovine system. Treatment of SRBC with 0·143 M AET for 15 min formed the highest number of rosettes with human lymphocytes (Pellegrino *et al.*, 1975), whereas SRBC treatment with 0·1 M AET for 20 min detected the highest percentage of RFC in bovine blood. As reported earlier (Grewal *et al.*, 1976), FCS has an enhancing effect on rosette formation by bovine lymphocytes (P. S. Paul, unpublished observations). However, 100% FCS was not required for optimum results in our assay. RPMI containing 10% FCS was found satisfactory. Different lots of FCS varied in their enhancing effect on rosette formation (P. S. Paul, unpublished data). Furthermore, absorption of most lots of FCS with SRBC was essential to remove non-specific agglutination of SRBC. Significant difference of various sources of SRBC on rosette formation was not observed with AET-treated SRBC as reported earlier using dextran (Wardley, 1977).

Two possible mechanisms on the enhancing effect of AET on rosette formation have been suggested by Pellegrino *et al.*, (1975). First, treatment of SRBC with AET may uncover sites on the cell membrane that are able to react with T cells or it may alter the physicochemical properties of SRBC surface so as to favour their attachment to the plasma membrane of reactive lymphocytes. The exact mechanism of action of AET, however, is unknown at the present time.

Methods for the identification and enumeration of bovine B lymphocytes have been extensively used (Muscoplat *et al.*, 1974; Rouse & Babiuk, 1974; Weiland & Straub, 1975; Paul *et al.*, 1977b; Kenyon & Piper, 1977; Kumar *et al.*, 1978; Grewal *et al.*, 1978); however, procedures for the identification and quantitation of bovine monocytes have not been developed. B lymphocytes bear easily detectable sIg and possess receptors for the third component of complement (C3) and Fc portion of immunoglobulins. Cells bearing sIg can be easily quantitated by staining with fluoresceinated anti-Ig, whereas cells possessing receptors for Fc and C3 can be enumerated by EA and EAC rosette assays, respectively. In the bovine, demonstration of sIg on lymphocytes is the most commonly used criterion for the identification of B lymphocytes. By this method, the percentage of B cells in peripheral blood of normal cattle has been reported to be in the range of 15–30% (Muscoplat *et al.*, 1974; Rouse & Babiuk, 1974; Kenyon & Piper, 1977; Kumar *et al.*, 1978). Since monocytes bear Fc receptors and are capable of mimicking B cells by adsorption of immunoglobulins through Fc receptors, we wished to develop a method for the differentiation of bovine B lymphocytes from monocytes. Latex phagocytosis is a simple procedure and offers a useful tool for differentiation of monocytes from B lymphocytes (Winchester & Ross, 1976). We have adapted this procedure to identify bovine monocytes. Furthermore, the normal precentage of B cells and monocytes in peripheral blood was determined by examining PBL from eleven normal cattle by latex phagocytosis and staining with anti-Ig, simultaneously. Approximately 10–30% PBL-ingested latex particles, whereas 30% of latex-ingesting cells were sIg- positive. This study showed that although 16% PBL were positive for sIg, 34% of these sIg^+ cells phagocytosed latex particles. Thus, the percentage of B lymphocytes (10·7%) in bovine was similar to that in man (Winchester & Ross, 1976). This study also demonstrates the importance of identification of monocytes for accurate determination of B cells.

Development of surface-membrane markers in man and animals has made it possible to enumerate T and B lymphocytes. This information has proved valuable in gaining a better understanding of roles of various cell types in health and disease (Wybran & Fudenberg, 1973; Townes & Postlethwaite, 1977; Terrell, Holmberg & Osburn, 1977). These markers have also been employed in immunological characterization of leukaemias in man (Preud'Homme & Seligmann, 1974; Townes & Postlethwaite, 1977) and animals (Hudson & Payne, 1973; Greenberg & Zatz, 1975; Cockeral *et al.*, 1976). With the availability of improved methodology for enumeration of bovine T and B lymphocytes in our laboratory, we proceeded to determine the percentages of various lymphoid populations in tissues and peripheral blood of normal cattle. In our study, 83–90% bovine thymocytes and 44–70% splenic lymphocytes formed rosettes with AET-treated SRBC. These findings are similar to those reported in man and guinea-pigs (Johansen *et al.*, 1974). In peripheral blood of twenty normal cattle, a mean of 63% T and 11% B cells (sIg^+ latex non-ingesting) were detected. Approximately 26% PBL did not contain markers for T and B cells and probably represent a null cell population. It is also possible that a portion of these cells are a subpopulation of T cells which do not form rosettes with AET-treated SRBC or are monocytes not detected by latex

phagocytosis. This data on normal values of lymphoid populations in peripheral blood of normal cattle should provide an excellent reference to compare with the values in diseased cattle.

Persistent lymphocytosis, a prodromal stage of bovine leukaemia, has been reported to be a B-cell abnormality as shown by increased percentage of sIg, complement receptor and Fc receptor bearing lymphocytes in peripheral blood (Muscoplat *et al.*, 1974; Kenyon & Piper, 1977; Kumar *et al.*, 1978). In our study, we have confirmed these findings and shown that B-cell percentages are elevated while T-cell percentages are decreased in peripheral blood of cattle with persistent lymphocytosis.

In this report, we have presented conclusive evidence that AET-treated SRBC bind to bovine T and not B lymphocytes. Our proof stems from experiments on simultaneous detection for sIg, a marker for B cells and rosette formation with AET-treated SRBC on lymphocyte subpopulations fractionated on nylon-wool columns. Nylon-wool-adherent cells were enriched in sIg^+ cells and contained a very few RFC, whereas nylon-non-adherent cells were depleted of sIg^+ cells and were enriched in RFC. Furthermore, none of the RFC had detectable sIg. A large percentage (83–90%) of thymocytes from three foetuses and two heifers formed rosettes with AET-treated SRBC, thus providing additional evidence that RFC are T cells. Evidence was also presented that these cells are not monocytes since none of the latex-ingesting cells formed rosettes.

In summary, we have described an improved assay for the detection of T lymphocytes in cattle. Furthermore, we have reported the percentages of T and B lymphocytes in tissues and peripheral blood of normal and lymphocytotic cattle. Identification and characterization of further subpopulations of bovine T and B lymphocytes and determination of their role in health and disease is underway.

This study was supported in part by Public Health service grant CA 17232 from the National Cancer Institute, the U.S. Department of Agriculture, Cooperative State Research Service research grant 701-15-41, and the Minnesota Agricultural Experiment Station.

REFERENCES

BACH, J.F., DORMONT, J.D., DARDENNE, M. & BALNER, H. (1968) *In vitro* rosette inhibition by antihuman antilymphocyte serum. Correlation with skin graft survival in subhuman primates. *Transplantation*, **8**, 265.

BOWLES, C.A., WHITE, G.S. & LUCAS, D. (1975) Rosette formation by canine peripheral blood lymphocytes. *J. Immunol.* **114**, 399.

BÖYUM, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation. *Scand. J. clin. Lab. Invest.* **21** (suppl. 97), 77.

BROWN, C.S., HALPERN, H. & WORTIS, H.H. (1975) Enhanced rosetting of sheep erythrocytes by peripheral blood T cells in the presence of dextran. *Clin. exp. Immunol.* **20**, 505.

COCKEREL, G.L., KRAKOWKA, S., HOOVER, E.A., OLSEN, R.G. & YOHN, D.S. (1976) Characterization of feline T and B lymphocytes and identification of an experimentally induced T cell neoplasm in the cat. *J. Nat. Cancer Inst.* **57**, 907.

ESCAJADILLO, C. & BINNS, R.M. (1975a) Rosette formation with sheep erythrocytes—A possible T-cell marker in the pig. *Int. Arch. Allergy Appl. Immunol.* **48**, 261.

ESCAJADILLO, C. & BINNS, R.M. (1975b) Rosette formation of pig T lymphocytes with sheep erythrocytes. *Int. Arch. Allergy Appl. Immunol.* **49**, 325.

FLOREY, M.J. & PEETOOM, F. (1976) Modified E-rosette test for detection of total and active rosette-forming lymphocytes. *J. Immunol. Methods.* **13**, 201.

GREENBERG, R.S. & ZATZ, M.M. (1975) Spontaneous AKR lymphoma with T and B-cell characteristics. *Nature (Lond)*, **257**, 314.

GREWAL, A.S., ROUSE, B.T. & BABIUK, L.A. (1976) Erythrocyte rosettes—a marker for bovine T cells. *Can. J. Comp. Med.* **40**, 298.

GREWAL, A.S., ROUSE, B.T. & BABIUK, L.A. (1978) Characterization of surface receptors on bovine leukocytes. *Int. Arch. Allergy Appl. Immunol.* **56**, 289.

HIGGINS, D.A. & STACK, M.J. (1977) Bovine lymphocytes: recognition of cells forming spontaneous (E) rosttes. *Clin. exp. Immunol.* **27**, 348.

HORWITZ, D.A., ALLISON, A. C., WARD, P., & KIGHT, N. (1977) Identification of human mononuclear leukocyte populations by esterase staining. *Clin. exp. Immunol.* **30**, 289.

HUDSON, L. & PAYNE, L.N. (1973) An analysis of the T and B cells of Marek's disease lymphomas of the chicken. *Nature: New Biology*, **241**, 52.

JOHANSEN, K.S., JOHANSEN, T.S. & TALMAGE, D.W. (1974) T cell rosette formation in primates, pigs, and guinea pigs. *J. Allergy Clin. Immunol.* **54**, 86.

JONDAL, M., HOLM, G. & WIGZELL, H. (1972) Surface markers on human T and B lymphocytes. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. *J. exp. Med.* **136**, 207.

KAPLAN, M.E. & CLARK, C. (1974) An improved rosetting assay for detection of human T lymphocytes. *J. Immunol. Methods*, **5**, 131.

KENYON, S.J. & PIPER, C.E. (1977) Cellular basis of persistent lymphocytosis in cattle infected with bovine leukemia virus. *Infec. Immunity*, **16**, 891.

KUMAR, S.P., PAUL, P.S., POMEROY, K.A., JOHNSON, D.W., MUSCOPLAT, C.C., VAN DER MAATEN, M.J., MILLER, J.M. & SORENSEN, D.K. (1978) Frequency of lymphocytes bearing Fc receptors and surface membrane immuno-

globulins in normal, persistent lymphocytotic and leukemic cows. *Am. J. Vet. Res.* **39**, 45.

LAY, W.H., MENDES, N.F., BIANCO, C. & NUSSENZWEIG, V. (1971) Binding of sheep red blood cells to a large population of human lymphocytes. *Nature (Lond.)*, **230**, 531.

MUSCOPLAT, C.C., JOHNSON, D.W., POMEROY, K.A., OLSON, J.M., LARSON, V.L., STEVENS, J.B. & SORENSEN, D.K. (1974) Lymphocyte surface immunoglobulins: ffequency in normal and lymphocytotic cattle. *Am. J. Vet. Res.* **35**, 593.

PAUL, P.S., POMEROY, K.A., JOHNSON, D.W., MUSCOPLAT, C.C., HANDWERGER, B.S., SOPER, F.F. & SORENSEN, D.K. Evidence for the replication of bovine leukemia virus in the B lymphocytes. (1977b) *Am. J. Vet. Res.* **38**, 873.

PAUL, P.S., POMEROY, K.A., CASTRO, A.E., JOHNSON, D.W., MUSCOPLAT, C.C. & SORENSEN, D.K. (1977a) Detection of bovine leukemia virus in B-lymphocytes by the syncytia induction assay. *J. Nat. Cancer Inst*, **59**, 1269.

PELLEGRINO, M.A., FERRONE, S., DIERICH M.P. & RIESFELD R.A. (1975) Enhancement of sheep red blood cell human lymphocyte rosette formation by the sulfhydryl compound 2-amino ethylisothiouronium bromide. *Clin. Immunol. Immunopathol.* **3**, 324.

PREUD'HOMME J.L. & FLANDRIN G. (1974) Identification by peroxidase staining of monocytes in surface immunofluorescence tests. *J. Immunol.* **113**, 1650.

PREUD'HOMME, J.L. & SELIGMANN, M. (1974) Surface immunoglobulin on human lymphoid cells. *Prog. clin. Immunol.* **2**, 121.

ROBERTS S.J. (1971) Fetal size and characteristics at various stages of pregnancy. In *Veterinary Obstetrics and Genital Diseases (Theriogenology)* ch. 2 pp. 19–20. Stephen J. Roberts Ithaca N.Y.

ROUSE, B.T. & BABIUK, L.A. (1974) Host responses to infectious bovine rhinotracheitis virus III. Isolation and immunologic activities of bovine T lymphocytes. *J. Immunol.* **113**, 1391.

SHIMIZU, M., PAN, I.C. & HESS, W.R. (1976) T and B Lymphocytes in porcine blood. *Am. J. Vet. Res.* **37**, 309.

TARR, M.J., OLSEN, R.G., KRAKOWKA, G.S., COCKERELL, G. L. & GABEL, A.A. (1977) Erythrocyte rosette formation of equine peripheral blood lymphocytes. *Am. J. Vet. Res.* **38**, 1775.

TERRITO, M.C., GOLDE, D.W. & CLINE, M.J. (1976) *Macrophage activation and function, Manual of Clinical Immunology* (ed. N. R. Rose and H. Friedman) ch. 16. American Society of Microbiology, Washington, D.C.

TERRELL, T.G., HOLMBERG, C.A., & OSBURN, B.I. (1977) Immunologic surface markers on nonhuman primate lymphocytes. *Am. J. Vet. Res.* **38**, 503.

TOWNES, A.S. & POSTLETHWAITE, A.E. (1977) Lymphocyte surface markers in human disease. *Adv. Intern. Med.* **22**, 97.

WARDLEY, R. (1977) An improved E rosetting technique for cattle. *Br. Vet. J.* **133**, 432.

WEILAND, F. & STRAUB, O.C. (1975) Frequency of surface immunoglobulin bearing blood lymphocytes in cattle affected with bovine leukosis. *Res. Vet. Sci.* **19**, 100.

WEINER, M.S., BIANCO, C. & NUSSENZWEIG, V. (1973) Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. *Blood*, **42**, 939.

WINCHESTER, R.J. & ROSS, G. (1976) *Methods for enumerating lymphocyte populations. Manual of Clinical Immunology* (ed. N. R. Rose and H. Freidman), ch. 7. American Society of Microbiology, Washington, D.C.

WOODY, J.N. & SELL, K.W. (1975) Characteristics of the 'active rosette test'. Technical considerations of the test and comments. *J. Immunol. Meth.* **8**, 331.

WYBRAN, J. & FUDENBERG, H.H. (1973) Thymus-derived rosette-forming cells in various human disease states: cancer, lymphoma, bacterial and viral infections, and other diseases. *J. clin. Invest.* **52**, 1026.

ZUCKER-FRANKLIN, D. (1974) The percentage of monocytes among 'mononuclear' cell fractions obtained from normal human blood. *J. Immunol.* **112**, 234.