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ENUMERATION AND ISOLATION OF HUMAN T AND B LYMPHOCYTES BY ROSETTE FORMATION WITH ANTIBODY-COATED ERYTHROCYTES*

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SUMMARY

Rosette techniques are presented for the enumeration and separation of both Ig^+ T^- and Ig^- T^+ human lymphocytes. In order to enumerate Ig^+ cells, the direct immunocytoadhesion technique was employed using human erythrocytes (E) coated with purified anti-κ or anti-λ light chain antibodies. Specificity of these rosettes was shown with chronic lymphocytic leukaemias of either the κ or λ type. T^+ cells were enumerated by a new indirect rosette technique in which the lymphocytes were initially treated with rabbit anti-human thymus cell antiserum followed by direct rosetting with human E coated with purified anti-rabbit light chain antibody. For normal individuals, 24–32% Ig^+ T^- cells and 65–71% Ig^- T^+ cells were found among the lymphocytes of peripheral blood as well as tonsils with these rosette methods. The Ficoll–Hypaque method was used to obtain purified Ig^- T^+ and Ig^+ T^- cells by removing rosetted Ig^+ cells or T^+ cells, respectively. The purity of the Ig^- T^+ cells was indicated by >99% indirect rosetting of cells sensitized with anti-human thymus cell antibody (Ab) and by less than 1% direct rosetting with anti-κ Ab-E+ anti-λ Ab-E. The purity of the Ig^+ T^- cells obtained was indicated by 92–96% direct rosetting with anti-κ Ab-E+anti-λ Ab-E and by less than 1% indirect rosetting with anti-human thymus cell antibody. A small percentage of Ig^- T^- 'null' cells could not be identified by either reagent. Thus, essentially pure Ig^- T^+ and Ig^+ T^- cells were readily and efficiently isolated by 'negative selection' thereby lessening the

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possibility of functional changes that may develop by more extensive manipulation of lymphocytes.

INTRODUCTION

Two main subpopulations of human lymphocytes have been distinguished: thymus-derived (T cells), which appear to be non-immunoglobulin bearing (Ig^-), and bone marrow-derived (B cells), which contain surface immunoglobulin (Ig^+) (see Greaves, Owen & Raff, 1973). B cells have been enumerated in human peripheral blood by immunofluorescence (Fröland, Natvig & Berdal, 1971; Grey, Rabellino & Pirofsky, 1971) as well as by rosette formation detecting Fc receptors (Dickler & Kunkel, 1972) or complement receptors (Ross *et al.*, 1973). In addition, B cells have been enumerated by rosette formation with anti-human $F(ab')_2$ antibody-coated erythrocytes (Giuliano *et al.*, 1974). T cells, until recently, have been enumerated by their ability to bind non-specifically to sheep erythrocytes and form E rosettes (Fröland, 1972; Jondal, Holm & Wigzell, 1972; Papamichail *et al.*, 1972; Wybran, Carr & Fudenberg, 1972). Also, enumeration of both B and T cells in human peripheral blood has been accomplished by using specific fluorescent anti-B and anti-T-cell antisera (Brown & Greaves, 1974).

Methods for the isolation of different subpopulations have been developed utilizing differences in density in albumin gradients, sedimentation velocities, electrophoretic mobilities (see Denman, 1973) and adhesion to nylon fibres (Eisen, Wedner & Parker, 1972; Greaves & Brown, 1973). The ability of B cells to bind column materials coupled with anti-Ig antibodies (Fröland *et al.*, 1971; Campbell & Grey, 1972; Schlossman & Hudson, 1973; Chess, MacDermott & Schlossman, 1974) has been used recently for T- and B-cell separation. Other separation methods were developed using rosette techniques followed by Ficoll–Hypaque gradient separation of rosetted cells, e.g. T cells forming E rosettes with sheep erythrocytes (SRBC) (Wybran, Chantler & Fudenberg, 1973) and B cells forming rosettes with antibody-coated erythrocytes (Molinaro *et al.*, 1975a). Starting from the observations that strong direct rosettes (Molinaro & Dray, 1974; Molinaro, Maron & Dray, 1974) and indirect rosettes (Molinaro *et al.*, 1975b) could be obtained with purified antibody coated to erythrocytes, we have developed simple rosette methods useful for the enumeration of Ig^+ T^- (B cells) and Ig^- T^+ (T cells) as well as their isolation by negative selection on Ficoll–Hypaque gradients. Ig^+ T^- cells were rosetted *directly* with purified anti-light chain antibodies coated to human erythrocytes. Ig^- T^+ cells were first sensitized with specific anti-human thymus cell antiserum made in b^4b^4 rabbits and then rosetted *indirectly* with purified anti-rabbit light chain allotype antibody (anti-b4) coated to human erythrocytes. Both Ig^+ T^- and Ig^- T^+ cells were enumerated with these techniques in normal individuals and patients. In addition, both purified Ig^- T^+ and Ig^+ T^- cells were isolated using these direct and indirect rosette techniques followed by Ficoll–Hypaque gradient separation.

MATERIALS AND METHODS

Lymphocytes. Peripheral blood lymphocytes were separated on Ficoll–Hypaque as described by Böyum (1968). Tonsil cells (obtained through the co-operation of Dr A. H. Andrews and the Ear, Nose and Throat Clinic, University of Illinois Medical Center, Chicago, Illinois) were passed through sterile stainless steel

mesh screens, filtered through sterile cotton and washed four times in Eagle's MEM (Grand Island Biologicals, Grand Island, New York).

Purified anti-light chain antibodies. Lyophilized purified Bence–Jones proteins of the kappa and lambda variety were gifts from Dr Paul Heller (West Side Veterans Administration Hospital, Chicago, Illinois). For immunization, 5 mg of protein were emulsified in Freund's complete adjuvant (Difco, Detroit, Michigan) and injected intramuscularly and into the foot pads. Rabbits were boosted 14 days later with intramuscular injections of 5 mg of each protein type emulsified in Freund's incomplete adjuvant.

The purified kappa and lambda chains were coupled to cyanogen bromide-activated Sepharose 4 B columns (Pharmacia, Piscataway, New Jersey) utilizing methods described by Axen, Porath & Ernback (1967). The columns were washed until the effluents had 0·01 OD units cm^{-1} at 280 nm. The antibodies were then eluted by glycine–H_2SO_4 buffer (0·15 M, pH 2·5) containing NaCl (0·35 M). The eluates were immediately neutralized with 0·15 M Tris base and dialysed against 0·15 M NaCl. Both purified antibody preparations were tested against whole human serum as well as for cross-reactivity against 10 mg of kappa or lambda chains by immunoelectrophoresis.

Preparation of anti-thymus cell antisera. Thymus tissue (obtained through the co-operation of the Department of Pathology, University of Illinois at the Medical Center), extirpated within approximately 8 hr post-mortem, was passed through a sterile stainless steel mesh screen, filtered through sterile cotton and washed four times in Eagle's MEM. Approximately 2×10^8 cells were emulsified in 2 ml of Freund's complete adjuvant and administered to b^4b^4 homozygous rabbits. Twenty-one days later 1×10^8 thoroughly washed thymus cells suspended in MEM were injected into the marginal ear vein on 3 consecutive days. Seven days later the rabbits were bled and the serum was tested on freshly collected lymphocytes for rosetting. Those rabbits whose sera exhibited an indirect rosetting titre of 1:10,000 or more were bled at 4-day intervals. The sera were aliquoted in 2·0 ml amounts and stored at 0°C. One-millilitre amounts of serum were adsorbed once with equal volumes of washed A, B and O type packed erythrocytes, twice with equal volumes of packed liver cells and twice with chronic lymphocytic leukaemia cells using methods previously described by Greaves & Brown (1974). The adsorbed serum was tested by indirect rosetting at different dilutions against purified Ig^- cells, total lymphocyte populations and chronic lymphocytic leukaemia cells.

Direct rosettes (Ig^+ T^- cells). Anti-kappa and anti-lambda antibodies were separately coupled to human type O erythrocytes (HuE) utilizing the chromium chloride method of Gold & Fudenberg (1967). The lymphocytes were mixed in an ice bath with either anti-kappa antibody-coated human erythrocytes (anti-κ Ab-HuE), anti-lambda antibody-coated human erythrocytes (anti-λ Ab-HuE), or equal volumes of both at a ratio of approximately twenty erythrocytes to one lymphocyte. This mixture was centrifuged at 1700 *g* for 5 min at 4°C. The pellet was resuspended by pipetting, stained with toluidine blue, and the percentage of rosettes was determined by microscopic examination of 200–300 cells.

Indirect rosettes (Ig^- T^+). Lymphocytes at a concentration of 2×10^6–8×10^6/ml were initially sensitized by incubation with equal volumes of anti-thymus cell antisera suspended in RPMI 1640 at the appropriate dilution at 0°C for 30 min and washed four times with MEM. They were then resuspended with HuE coated with antibody specific for rabbit light chains (anti-b4 Ab-HuE) at a ratio of twenty erythrocytes to one lymphocyte. This mixture was centrifuged at 1700 *g* for 5 min at 4°C. The pellet was resuspended by pipetting, stained with toluidine blue and the percentage of rosettes was determined by microscopic examination of 200–300 cells.

Erosettes. Lymphocytes were mixed with thoroughly washed sheep erythrocytes at a ratio of approximately a hundred erythrocytes to one lymphocyte, centrifuged at 800 *g* for 10 min, and very gently resuspended. The cells were stained with toluidine blue and the percentage of rosettes was determined by microscopic examination of 200–300 cells.

Ficoll–Hypaque gradient separation of direct or indirect rosetted lymphocytes. One millilitre of either the direct or indirect rosetted mixture was layered on to 2·0 ml of Ficoll–Hypaque and centrifuged at 800 *g* for 20 min at 4°C. After centrifugation, the cells at the interface were collected and tested by either direct or indirect rosetting to determine the content of Ig^+ T^- and Ig^- T^+ cells.

RESULTS

Enumeration of Ig^+ cells by direct rosetting

Ig^+ cells were rosetted with HuE coated with antibody to either human kappa (anti-κ

Ab-HuE) or human lambda (anti-λ Ab-HuE). The specificity of the anti-κ Ab-HuE and anti-λ Ab-HuE was determined by rosetting with lymphocytes of monoclonal leukaemias of known light chain type. The anti-λ Ab-HuE rosetted all of the lymphocytes bearing surface immunoglobulin of patients Gol and Her (λ-type chronic lymphocytic leukaemia) and essentially none of those of patient Cze (κ-type chronic lymphocytic leukaemia). On the other hand, the anti-λ Ab-HuE rosetted all of the lymphocytes bearing surface immunoglobulin of patient Cze but none of those of Gol or Her (Table 1). The majority of specific rosettes generally have four or more indicator erythrocytes bound to each lymphocyte (Fig. 1). These results are concordant with those obtained with fluorescent antibody data pertaining to these patients (personal communication from Dr Paul Heller, West Side Veterans Administration Hospital, Chicago, Illinois). Lymphocytes from twelve normal donors were tested for direct anti-light chain rosettes using a mixture of anti-κ and anti-λ Ab-HuE. The percentage of Ig^+ cells in normal donors varied between 24 and 32% (Table 2).

TABLE 1. Direct rosettes with lymphocytes from chronic lymphocytic leukaemia patients with human erythrocytes (HuE) coated with anti-kappa antibody (anti-κ Ab-HuE), anti-lambda antibody (anti-λ Ab-HuE) or equal mixtures of both

Donors	White blood cell count	Rosettes with Ab-HuE specific for: $\kappa+\lambda$ (%)	κ (%)	λ (%)
Gol	224,500	81	0	82
Her	48,500	75	1	73
Cze	n.d.	72	74	1

n.d. = Not determined.

TABLE 2. Direct rosettes of normal human peripheral lymphocytes with a mixture of anti-κ Ab-HuE plus anti-λ Ab-HuE

Donors	White blood cell count (number/mm^3)	Ig^+ rosettes ($\kappa+\lambda$)
Bie	7510	26
Ton	4450	25
3248†	2670	25
Kat	10,600	29
Ela	7750	24
Mit	5650	27
Ket	5050	31
Teo	n.d.	32
Gem	n.d.	31
Bar	n.d.	28
Mar	n.d.	29
Fxj*	n.d.	31

* Tonsil cells.

† Blood donor, University of Illinois Hospital Blood Bank.

Isolation of Ig^- cells by Ficoll separation of direct rosettes

Lymphocytes were obtained from 20 ml of blood collected from seven normal donors. In addition, tonsil cells from one individual were used. The percentage of Ig^- cells was determined for each individual after direct rosetting of Ig^+ cells prior to separation. After resuspending, the rosetted mixture was layered on to Ficoll and centrifuged. The Ig^- cells remaining at the Ficoll–Hypaque interface were collected and counted. The number of contaminating Ig^+ cells was less than 1·0% when tested by re-rosetting with a mixture of

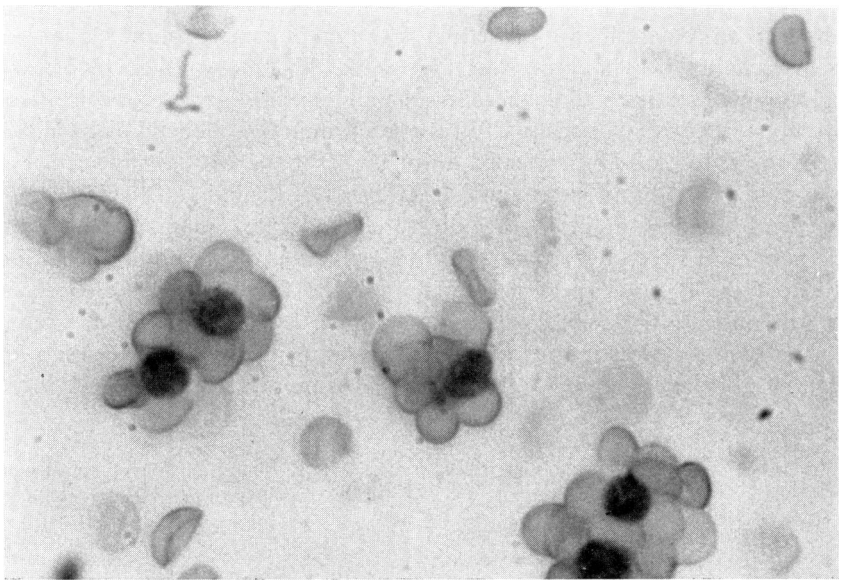

FIG. 1. Human Ig^+ lymphocytes directly rosetted with mixtures of antibody-coated human erythrocytes (Ab-HuE) specific for κ (anti-κ Ab-HuE) or λ (anti-λ Ab-HuE) human light chains. (Magnification × 560.)

Ab-HuE specific for human κ and λ light chains. The results with the tonsil cell preparation was similar to those obtained with peripheral blood (Table 3). The yield of Ig^- cells relative to the total number of Ig^- cells in the unseparated population (Table 3) varied from 48 to 80%.

TABLE 3. Isolation of purified Ig^- cells by direct rosetting with mixtures of anti-κ Ab-HuE plus anti-λ Ab-HuE

		Ig^- cells	
Experiment number	Donors	Yield of Ig^- cells (%)	Re-rosetted with anti-light chain-coated HuE (%)
1	Bil	74	0·5
1	Ton	56	0·5
2	Ket	58	0·1
2	Teo	48	0·3
2	Gen	62	0·1
2	Bar	80	0·1
2	Mar	76	0·1
2	Fxj*	69	0·3

* Tonsil lymphocytes.

Enumeration of T^+ cells by indirect rosetting

Adsorbed rabbit anti-human thymus cells antisera was tested for activity by indirect rosetting at different dilutions against rosette-purified Ig^- cells, chronic lymphocytic leukaemia cells and normal unseparated lymphocytes of two donors. The antiserum at a dilution of 1:16 was effective in sensitizing Ig^- but not Ig^+ cells for rosette formation with Ab-HuE specific for rabbit light chains; thus, 98% of purified Ig^- cells, 65–67% of unseparated lymphocytes, and 17% of lymphocytes from a chronic lymphocytic leukaemia patient (Gol, Table 1) who was known to have 81% B cells were rosetted (Fig. 2). The dilution of

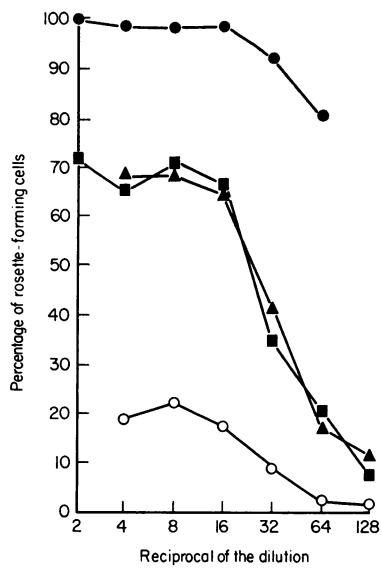

FIG. 2. Adsorbed anti-human thymus cell antisera was diluted in RPMI 1640. Equal volumes of the diluted antisera were added to lymphocyte populations at a concentration of 2×10^6/ml as follows: (a) to purified Ig^- cells (●); (b) unseparated lymphocytes from two normal donors (Bil (■) and Ton (▲); (c) unseparated lymphocytes from a chronic lymphocytic leukaemia patient (Gol) (○) who was shown to have 82% Ig^+ cells. The cells were incubated at 0°C for 30 min and washed four times with MEM. The cells were then resuspended with human erythrocytes coated with antibody specific for rabbit light chains at a ratio of twenty erythrocytes to one lymphocyte. The pellet was resuspended by pipetting, stained with toluidine blue and the percentage of rosettes was determined by microscopic examination of 200–300 cells.

antisera to achieve this discrimination of Ig^+ from Ig^- cells differed for each newly adsorbed aliquot of anti-human thymus cell antisera. When sheep red blood cells (SRBC) were used instead of HuE for indirect rosetting the shape of the curve, shown in Fig. 2, differed in that the plateau was less distinct; nevertheless, Ab–SRBC were also effective in discriminating between Ig^+ and Ig^- cells.

Peripheral blood lymphocytes of three normal individuals and of two patients with chronic lymphocytic leukaemia were treated with anti-human thymus cell antisera and then rosetted with anti-b4-coated HuE or SRBC (anti-b4 is specific for the light chain allotype of the anti-human thymus cell antibody). The results of these tests were compared with E rosette

data obtained for each individual. The values obtained by E rosettes were far less than those obtained by indirect rosetting in all individuals including the two chronic lymphocytic leukaemia patients (Table 4).

TABLE 4. Comparison of E rosettes with indirect (T^+) rosettes

Name	E rosettes (%)	Indirect rosettes (%)
Ton	54	66
Any	54	65
Bil	49	71
Gol*	9	17
Her*	11	24

* Chronic lymphocytic leukaemia patients.

Isolation of Ig^+ cells by Ficoll separation of anti-human thymus cell indirect rosettes

Ig^+ cells were isolated from peripheral blood lymphocytes of seven normal donors as well as tonsil lymphocytes of two patients. For this, the cells were incubated with anti-human thymus cell antisera at the appropriate dilution for 30 min at 0°C, washed four times in Eagle's MEM, rosetted with Ab-HuE specific for rabbit light chains and then layered on Ficoll and centrifuged. The cells remaining at the interface were collected, counted and examined by direct rosetting with Ab-HuE specific for human light chains and were shown to vary in the ability to rosette with anti-light chain-coated erythrocytes, between 92 and 96%. The cells were also tested for by indirect re-rosetting using anti-human thymus cell antisera sensitization followed by rosetting with Ab-HuE specific for rabbit light chains; in all individuals less than 1·0% of these cells re-rosetted. The percentage of Ig^+ cells in unseparated populations varied from 24 to 32% based on direct rosetting with Ab-HuE specific for human light chains. The yield of Ig^+ cells relative to the total number of Ig^+ cells in the unseparated population (Table 5) varied from 52 to 74%. Essentially, the same results were obtained when sheep red blood cells were used instead of HuE for rosetting.

DISCUSSION

We have used antibody-coated erythrocytes (Ab-E) to develop rapid and efficient immunocytoadhesion techniques for the enumeration and isolation of Ig^+ T^- and Ig^- T^+ lymphocytes. Mixtures of anti-human κ Ab-HuE and anti-human λ Ab-HuE were used to enumerate Ig^+ cells by direct rosetting. Anti-rabbit light chain Ab-HuE were used to enumerate T^+ cells by indirect rosetting of T^+ cells after their sensitization with rabbit anti-human thymus cell antibody. The isolation of Ig^- T^+ or Ig^+ T^- cells was done by removal of rosetted Ig^+ or T^+ cells, respectively, in a Ficoll–Hypaque density gradient.

The specificity of Ig^+ cell rosettes was demonstrated by the use of lymphocytes from κ- or λ-type leukaemias. The specificity of our anti-human thymus cell antiserum is supported by the following W.H.O. technical report criteria (Aiuti *et al.*, 1974): (a) this reagent can

TABLE 5. Isolation of Ig^+ cells using anti-thymus cell indirect rosettes

Experiment number	Donors	Yield of Ig^+ cells (%)	Top cells rosetted with anti-κ Ab-HuE plus anti-λ Ab-HuE (%)	Indirect re-rosetting (%)
1	Bxm*	63	94	0·6
1	Fxj*	67	93	0·9
2	Ton	60	94	0·4
2	Rog	62	96	0·7
2	Ana	67	93	0·3
2	Jen	68	93	0·4
2	Dan	74	96	0·5
2	Bic	52	95	0·8
2	Ket	63	92	0·6

* Tonsil lymphocytes.

be titrated to a plateau which defines a distinct lymphocyte population; (b) it will sensitize the majority of human lymphocytes, almost all lymphocytes after depletion of Ig^+ cells, but it will not react with the majority of typical chronic lymphocytic leukaemia cells (Fig. 2). It is important to note that the dilution at which the antiserum is used is a function of the adsorption procedure and type of antibody-coated erythrocytes and each newly adsorbed antiserum sample must be titrated, as illustrated in Fig. 2, before being used in the separation procedure.

Our method of isolation of Ig^+ T^- and Ig^- T^+ lymphocytes is by 'negative selection' since specific reagents are not bound to the cells selected for isolation. This method meets the main requirement of minimal manipulation of cells stressed by a recent W.H.O. technical report (Aiuti *et al.*, 1974). This should minimize functional abnormalities which might result from excessive manipulation of the cells, as in methods employing immunoadherence to columns which require that the Ig^+ cells adhering to the column must be subsequently removed. The question of possible impairment of functional capabilities after this removal process from the columns remains to be completely documented. We are as yet unable to eliminate the possibility that a few anti-human thymus antibody molecules may also bind to Ig^+ cells; however, the number of such antibody molecules were not great enough to form a rosette with anti-rabbit light chain Ab-E. The Ig^- T^+ cells collected at the interface in our experiments appear to be functionally normal with respect to their response to antigen and mitogen stimulation (unpublished observations).

The proportions of Ig^+ cells which we found among peripheral blood lymphocytes of normal individuals are in accord with those found by others with techniques such as immunofluorescence (Greaves & Brown, 1974) and anti-human $F(ab')_2$ coated sheep erythrocytes (Giuliano *et al.*, 1974). We obtained higher values for T^+ cells by our indirect method with Ab-HuE than we were able to obtain using the E rosette technique. These indirect rosette values were also higher than the E rosette values recently reported by Heier (1974). Thus, the indirect rosettes of T^+ cells were much more stable than E rosettes and therefore their enumeration was more sensitive.

In isolating $Ig^- T^+$ or $Ig^+ T^-$ cells, the yield of purified cells was only moderate varying from 48 to 80% for $Ig^- T^+$ cells and between 52 and 74% for $Ig^+ T^-$ cells. However, hardly any detectable contamination of $Ig^- T^+$ cells (<1%) with $Ig^+ T^-$ cells was observed. The purified Ig^+ cells were shown to be only 92–96% Ig^+, however, less than 1% were able to be indirectly rosetted after resensitization with anti-human thymus cell antiserum. These values indicate that the purity of the $Ig^+ T^-$ and $Ig^- T^+$ cell populations is similar to those reported by Chess and co-workers (1974). The yield of purified cells would be considered low when compared to those obtained recently by affinity column methods (Chess *et al.*, 1974). However, the number of cells recovered is ample for culturing them and investigating their properties. In addition, the ease, efficiency and rapidity of our method coupled with the element of negative selection provides some practical advantages.

The values obtained using our methods indicate that human peripheral blood lymphocytes isolated by Ficoll–Hypaque consist of approximately 28% Ig^+ cells, 68% T^+ cells and approximately 6% that appear to be $Ig^- T^-$ 'null' cells. Since monocytes were recently shown to represent 12% of mononuclear cells isolated by the Böyum method coupled with Ficoll–Hypaque density gradient centrifugation (Brown *et al.*, 1974), they should be found in the $Ig^+ T^-$ or $Ig^- T^+$ populations or in both. Preliminary experiments utilizing latex particle ingestion indicate that our $Ig^+ T^-$ cell population consists not only of B cells but also of approximately 50% monocytes. However, no monocytes were found among the $Ig^- T^+$ population.

Thus, we have developed direct and indirect rosette techniques which are sensitive and reproducible as well as efficient and inexpensive and which can be used to enumerate and isolate human $Ig^+ T^-$ cells or $Ig^- T^+$ cells. These isolated $Ig^+ T^-$ and $Ig^- T^+$ cells can then be assessed for their functional behaviour, e.g. response to antigens or mitogens.

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