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BRIEF COMMUNICATION

Enumeration of T and B lymphocytes in whole peripheral blood: absence of a null cell population

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SUMMARY

Alkaline phosphatase-labelled F(ab')$_2$ polyvalent anti-human immunoglobulin stained a mean of 12% (s.d. 4·2) of lymphocytes in the whole peripheral blood of 15 normal individuals. However, when the sensitivity of detection of the bound anti-immunoglobulin reagent was enhanced by adding complexes of alkaline phosphatase with F(ab')$_2$ anti-alkaline phosphatase, a mean of 22·1% (s.d. 7·8) of lymphocytes were positive. The mean number of T lymphocytes demonstrated in the same blood samples using a monoclonal anti-T lymphocyte antibody (OKT3) was 78% (s.d. 4·1) and was not increased by immunoenzyme enhancement. In five individuals the blood was washed at 37°C to remove passively adsorbed IgG and was then studied using the enhanced method together with monoclonal anti-κ and anti-λ antibodies. The mean $\pm$ s.d. number of κ-positive lymphocytes was 15·5 $\pm$ 4·6% and of λ-positive was 7·9 $\pm$ 1·1%. The sum of these was the same as the number of cells stained either with anti-κ and anti-λ together or with the conventional polyvalent anti-immunoglobulin, confirming that the enhancement procedure was detecting integral membrane immunoglobulin and not passively adsorbed IgG. Application to the same blood sample of both the anti-T cell antibody and the enhancement procedure with polyvalent anti-immunoglobulin stained 99–100% of lymphocytes. The present observations confirm that there are two populations among the B lymphocytes, the B-major cells with readily demonstrable surface immunoglobulin and the B-minor cells on which surface immunoglobulin is demonstrable only by very sensitive techniques (Haegert & Coombs, 1979). The B-major and B-minor cells together account for all the non-T lymphocytes and there are virtually no so-called 'null' cells in normal peripheral blood. These findings have significant implications for the use of surface membrane immunoglobulin as a marker in the typing of normal and abnormal lymphocyte populations.

INTRODUCTION

The defining marker of B lymphocytes is the presence of intrinsic surface membrane immunoglobulin which is readily detectable by a variety of different techniques of which the most widely used is direct immunofluorescence (Aiuti *et al.*, 1974). However Haegert & Coombs have recently demonstrated that, in addition to these classical B cells, there exists in human blood a population of cells on which surface immunoglobulin is not detectable by immunofluorescence, but which are positive when highly sensitive rosetting assays are used (Haegert, Hurd & Coombs, 1978; Haegert, 1978a). We now report similar findings using an immunoenzyme technique which is applicable to

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whole, unseparated blood and therefore permits precise enumeration of true circulating numbers of different lymphocyte populations (Druguet & Pepys, 1977). Furthermore, use in this method of a potent monoclonal antibody specific for a surface antigen on human peripheral T cells (Reinherz *et al.*, 1979) permitted us to establish normal values for the circulating proportions of T lymphocytes as well as B-major and B-minor populations.

MATERIALS AND METHODS

Processing of blood samples. Venous blood taken into EDTA from 15 normal healthy adult volunteers (six female, nine male), aged 22–50 years, was processed for the alkaline phosphatase–whole blood method of detecting lymphocyte surface markers as described elsewhere (Druguet & Pepys, 1977; Pepys & Pepys, 1980). The basis of this technique is that lymphocyte surface markers are labelled with alkaline phosphate-conjugated reagents (see below) in suspensions of washed whole blood. Permanent fixed smears are then made and stained for both alkaline phosphatase activity and for endogenous myeloperoxidase activity. The former reveals the labelled lymphocytes, the latter identifies and distinguishes monocytes and polymorphs. Counts of alkaline phosphatase-positive lymphocytes and of total lymphocytes provide both the percentage and, if required, the absolute number of cells bearing each surface marker. The only difference from earlier work was that all alkaline phosphatase–antibody conjugates were prepared using SPDP (Pharmacia GB Ltd, Middlesex, UK). Two milligrams of rabbit, sheep or goat $F(ab')_2$ were reacted with 40 μl of 5 mM SPDP, and 5 mg of alkaline phosphatase (type IIIS, Sigma Chemical Co. Ltd, Surrey, UK) were reacted with 125 μl of 5 mM SPDP according to the manufacturer's instructions (Pharmacia). The antibody reagent was then reduced with dithiothreitol (DTT) and conjugation performed as described by Pharmacia. These conjugates retained significantly higher antibody activity than those made with glutaraldehyde (Druguet & Pepys, 1977). All reagents were titrated in preliminary experiments and were subsequently used in dilutions at least two-fold lower than gave maximal staining. All slides were counted 'blind' by the same observer (E.O.P.).

Detection of T cells. Monoclonal antibody reactive with all peripheral T lymphocytes (OKT3, Ortho Pharmaceutical Corporation, Raritan, New Jersey, USA) was used at 20 μg/ml and was detected by alkaline phosphatase-labelled $F(ab')_2$ goat anti-mouse IgG.

Detection of B cells. Alkaline phosphatase-labelled rabbit $F(ab')_2$ anti-human IgG, with both anti-light chain and anti-γ activity, was used as before (Druguet & Pepys, 1977). Enhanced sensitivity for detection of cell surface immunoglobulin was achieved by including complexes of alkaline phosphatase with rabbit $F(ab')_2$ anti-alkaline phosphatase antibody in the incubation mixture. Rabbit antiserum to *E. coli* alkaline phosphatase (type IIIS, Sigma Chemical Co. Ltd, Surrey, UK) was raised as described previously (Pepys & Pepys, 1980) and the IgG fraction was isolated on Sepharose–protein A (Pharmacia GB Ltd, Middlesex, UK) (Goding, 1976). The IgG was digested for 16 hr at 37°C with 2% w/w pepsin (Sigma) at pH 4·0 and the resultant $F(ab')_2$ was freed of residual IgG and *p*Fc by a further passage over Sepharose–protein A (Goding, 1976). The absence of intact IgG was confirmed by double immunodiffusion analysis against sheep anti-rabbit IgG serum (provided by Dr A. Feinstein, ARC, Babraham) and by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. An optimal proportions titration was performed between alkaline phosphatase at 100 μg/ml in saline and $F(ab')_2$ anti-alkaline phosphatase. Enhancement of B cell staining was sought using complexes of various concentrations of alkaline phosphatase and doses of $F(ab')_2$ anti-alkaline phosphatase between optimal proportions and eight-fold antibody excess. Maximum numbers of cells were stained when 2·5 μg of alkaline phosphatase were mixed with a three-fold excess of antibody, incubated for 10 min at room temperature, and then added to the blood together with the alkaline phosphatase-labelled anti-human IgG. After 30 min of incubation at 4°C the blood was then washed and processed as described (Druguet & Pepys, 1977). Incubation of blood with these complexes as a separate step, after previous application of the labelled anti-immunoglobulin and washing, also enhanced staining but to a lesser extent than the one-stage procedure described above. The one-stage procedure was therefore adopted for subsequent use and was also tested in the final reaction step for detection of T cells and C3b receptors.

Monoclonal mouse anti-human κ light chain (Cat. No. 9470 SA) and anti-human λ light chain (Cat. No. 9472 SA) were obtained from the Molecular Diagnostics Division of Bethesda Research Labs Inc., Rockville, Maryland, USA, and were used at dilutions of 1/200 and 1/100 respectively. Since these reagents were in the form of ascitic fluid not $F(ab')_2$ fragments and the anti-κ was itself an IgG antibody, the blood to be tested was incubated for 10 min at 37°C in phosphate-buffered saline, pH 7·4, containing 0·2% w/w bovine serum albumin (Sigma) and then washed in this buffer at 37°C rather than 4°C before use (Pepys *et al.*, 1976). Cells binding the anti-light chain reagents were detected using alkaline phosphatase-labelled goat $F(ab')_2$ anti-mouse immunoglobulin together with the enhancement procedure.

Detection of C3b receptors. Complement receptor lymphocytes were detected as previously described (Pepys & Pepys, 1980).

RESULTS

Immunoenzyme enhancement of lymphocyte surface membrane staining

Maximum enhancement of staining was achieved by including $F(ab')_2$ anti-alkaline phosphatase–alkaline phosphatase complexes, formed in antibody excess, together with the covalently coupled alkaline phosphatase-labelled reagents in either direct or indirect tests. Inclusion of the complexes produced more intense staining of positive cells in tests for both C3b receptors and T cell-specific antigen, but it did not affect the number of positive cells (Table 1). In contrast, enhancement of staining for surface membrane immunoglobulin increased both the intensity of staining and the number of cells which were positive (Table 1).

Effect of immunoenzyme enhancement of staining for lymphocyte surface membrane immunoglobulin

The percentage of lymphocytes in whole normal human peripheral blood which stained in direct tests with alkaline phosphatase-labelled rabbit $F(ab')_2$ anti-IgG, containing anti-light chain and anti-γ specificities, was not significantly different from that we have previously reported (Table 1) (Druguet & Pepys, 1977). However, enhancement of the staining by inclusion of alkaline phosphatase–$F(ab')_2$ anti-alkaline phosphatase complexes demonstrated an increased number of positive cells (Table 1). The specificity for surface immunoglobulin of the staining of these additional lymphocytes was established by the fact that no staining at all was observed when the anti-IgG reagent was omitted nor when the anti-IgG reagent was absorbed with isolated IgG before use. In order to confirm that the enhanced procedure stained cells with their own integral surface membrane immunoglobulin rather than cells bearing immunoglobulin passively adsorbed from the plasma, blood was first incubated and washed at 37°C, which elutes such passively adsorbed

Table 1. Immunoenzyme enhancement of staining for B cells, T cells and C3b receptors

	Per cent lymphocytes positive (mean ± s.d.)					
	B cells		T cells		C3b receptor lymphocytes	
Number of subject	Standard test (B-major cells)	Enhanced test (B-major + B-minor cells)	Standard test	Enhanced test	Standard test	Enhanced test
15	12·0 ± 4·2	22·1 ± 7·8	78·0 ± 4·1	n.d.	n.d.	n.d.
7	n.d.	n.d.	75·1 ± 6·4	75·3 ± 4·7	6·2 ± 1·2	6·7 ± 1·6

n.d. = Not done.

immunoglobulin, and then tested with monoclonal anti-human κ and λ antibodies. Binding to lymphocytes of these mouse antibodies was demonstrated by incubation with alkaline phosphatase-labelled goat F(ab′)$_2$ anti-mouse immunoglobulin together with the alkaline phosphatase–F(ab′)$_2$ anti-alkaline phosphatase complexes. The ratio of κ to λ-bearing lymphocytes was approximately 2:1 (Table 2); furthermore, the sum of κ-positive and λ-positive cells detected separately was the same as the number detected either by using the two reagents together or by direct testing with the polyvalent anti-immunoglobulin reagent (Tables 1 and 2).

Table 2. Distribution of cells bearing κ and λ among the whole B lymphocyte population

	Per cent lymphocytes positive after immunoenzyme-enhanced staining with:			
Subject	Anti-λ†	Anti-κ†	Anti-$\lambda+\kappa$‡	F(ab′)$_2$ anti-human Ig§
E.O.	8·0	15·9	n.d.	n.d.
G.T.	8·0	13·0	n.d.	n.d.
M.L.	7·0	12·5	n.d.	n.d.
F.C.	9·7	23·3	33	34·3
M.B.	7·0	12·6	20·9	21·7
Mean ± s.d.	7·9 ± 1·1	15·5 ± 4·6		

Total mean λ-positive + mean κ-positive lymphocytes = 23·4%.

† Monoclonal mouse anti-human light chain antibodies.

‡ Mixture of monoclonal mouse anti-human κ and λ antibodies.

§ Rabbit F(ab′)$_2$ anti-human IgG with anti-light chain and anti-γ activity.

n.d. = Not done.

Normal proportions of T and B lymphocyte populations in the circulation

The mean proportion of lymphocytes stained by the monoclonal anti-human T cell reagent, regardless of immunoenzyme enhancement, was 78% whilst enhancement of the staining for surface immunoglobulin demonstrated a mean of 22% of positive cells (Table 1). Sequential application to the same blood sample of both the enhanced procedure for immunoglobulin and the anti-T cell reagent stained 99–100% of lymphocytes in each of eight different individuals. This indicates that the populations which bind the two sets of reagents are discrete and non-overlapping and together comprise virtually all the circulating lymphocytes.

DISCUSSION

A subset of normal peripheral lymphocytes is demonstrated here which fail to stain directly with labelled anti-immunoglobulin but on which surface immunoglobulin is detectable if a sufficiently sensitive technique is used. This confirms the observations of Coombs & Haegert who have described a B-major population, corresponding to the classical surface membrane immunoglobulin-bearing lymphocytes detected by immunofluorescence, and a B-minor population demonstrable only by the very sensitive mixed antiglobulin and direct antiglobulin rosetting methods (Haegert & Coombs, 1979; Auiti *et al.*, 1974; Haegert, 1978b; Coombs *et al.*, 1977). They also showed that the sum of sheep erythrocyte-rosetting and immunoglobulin-bearing cells was 100% and that by double labelling less than 5% of lymphocytes were both antiglobulin and sheep erythrocyte rosette-positive (Haegert *et al.*, 1978; Haegert, 1978b; Haegert, 1978a). Furthermore, after surface immunoglobulin

molecules had been enzymatically stripped, all immunoglobulin rosette-positive lymphocytes completely re-expressed their surface immunoglobulin determinants during *in vitro* culture (Haegert, 1978b).

We have not repeated these stripping experiments in the present study, but our previous experience and that of others shows that $F(ab')_2$ anti-IgG antibodies do not stain the 'labile' IgG which adheres to lymphocyte Fc(γ) receptors at 4°C and is eluted at 37°C (Lobo, Westervelt & Horwitz, 1975; Winchester *et al.*, 1975; Lobo & Horwitz, 1976; Pepys *et al.*, 1976). This is apparently because the staining of such 'labile' IgG depends upon its being stripped from the Fc(γ) receptors by anti-Fc antibodies, with the formation of IgG–anti-IgG complexes which then re-adhere to the Fc(γ) receptors via the Fc piece of the labelled antibody which remains available. All the antibody reagents used in the present enhancement procedure with polyvalent anti-immunoglobulin were $F(ab')_2$ preparations and the immunoglobulin detected on the so-called B-minor cells must therefore represent an intrinsic component of their own plasma membranes. This was confirmed by experiments using the enhancement method together with monoclonal antibodies specific for human κ and λ light chains respectively to stain lymphocytes in blood which had been incubated and then washed at 37°C. It is well established that such washing removes IgG which has been adsorbed passively from the plasma. The proportions of lymphocytes stained by anti-κ and anti-λ were in the ratio of 2:1 as previously reported by others (Aiuti *et al.*, 1974) and their sum was the same as the proportion of cells stained either by a mixture of the two monoclonal reagents or by polyvalent anti-IgG. The cells stained for κ and λ chains were therefore discrete, non-overlapping populations which together formed the global B-major plus B-minor population.

Separation of lymphocytes from blood, which is necessary for rosette testing, inevitably entails some loss of cells, particularly T cells, which may distort the true proportions of different populations in the circulation (Brown & Greaves, 1974). Our results with a method which avoids such losses (Druguet & Pepys, 1977) indicate that all the circulating lymphocytes are accounted for by T cells (78%) and the whole B cell population, B-major plus B-minor (22%). The absence of any 'null' or 'third' lymphocyte population and of any significant number of immunoglobulin-positive T cells was confirmed directly by double-labelling studies using the anti-T cell reagent together with the enhancement procedure for detection of surface immunoglobulin. This stained 99–100% of lymphocytes. Small monocytes which may in the past have been identified as 'null' lymphocytes by other authors were excluded in the present study by the use of intracellular peroxidase as a monocyte marker (Druguet & Pepys, 1977).

These observations have significant implications for the routine use of surface membrane immunoglobulin as a marker for B cells. In some lymphocytic leukaemias we have noted marked differences in the number of cells positive for immunoglobulin in the standard and enhanced tests; for example, 62% increasing to 87% in a case of hairy cell leukaemia, 54% rising to 92% in a case of prolymphocytic leukaemia and 65% rising to 90% in a case of chronic lymphocytic leukaemia. We therefore suggest that in studying both normal and abnormal lymphocytes it is advisable to include a technique of sufficient sensitivity to detect B-minor cells.

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