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The immuno-histochemical differentiation of human lymphoid cells

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rounds. Unfortunately, the majority of fetuses were aborted about 2.00 a.m. the following morning. Thus, of the first 35 fetuses examined, only two had a thymocyte viability of over 50%. However through the co-operation of ward staff an irregular supply of early aborted and therefore viable material was established, and a substantial stock of frozen thymocytes was built up. The thymus was removed and dissected free of blood vessels and connective tissue in sterile saline. Chopped material was pressed through a brass sieve into BSS. The sieves were made by welding mesh (4 holes/mm) onto the end of 3 cm plastic tubing with epoxy cement. The sieve was cleaned and sterilised after use with hydrogen peroxide (10 vol.). The thymocyte suspension was washed in BSS and examined for viability and concentration as detailed in sections 11.1.2. It was noted that the pellet from the first centrifugation could not be resuspended if the thymocytes were dead.

11.4 Lymphocyte storage by freezing

Techniques for successful freezing of cell suspensions have been well documented in the literature and few difficulties were encountered in the present work (Wood et al., 1965; Stopford et al., 1972; Strong et al., 1975).

Technique No.2 The preparation of live frozen lymphocytes for storage.

1) A cryoprotectant was made up as follows;

   20% dextrose solution: 3 parts

   Dimethyl sulphoxide (BDH): 2 parts.

2) The mixture was sterilised by passing it through

a 0.45 µl "Millipore" filter in a syringe adaptor and stored at 4°C.

3) The lymphocyte suspension was made upto the required concentration with 50% fetal calf serum in BSS. (Technique No.1)

4) One quarter of this volume of DMSO/dextrose solution was added, ensuring that all reagents and tubes were chilled on ice to prevent damage to lymphocytes by warm DMSO.

5) 2.0 ml volumes were dispensed into cold 3.0 ml glass vials and sealed in a flame.

6) The vials were identified and placed in a -70°C cabinet as soon as possible.

Lymphocytes were recovered by rapid melting in warm water followed by transfer to large volumes of BSS for centrifugal washing. PBL and CLL lymphocytes were successfully stored by this technique with loss of only a few percent viability and retention of functional marker properties for up to six months. Some loss of viability was experienced in thymocyte batches, but as fetuses had frequently been dead for some hours this loss was expected. The data on freeze preservation is presented in Table 2.

12 LYMPHOCYTE MARKER SYSTEMS

A battery of tests had to be developed to permit characterisation of cell suspensions, and to identify lymphocytes in mixed populations and tissue sections. The techniques were taken directly from the literature, and once successfully working, were strictly adhered to in order to ensure

Table 2 Data from records of frozen lymphocyte bank.

Product	Concentration per ml.	Viability %	Days Duration	Recovery Viability%
PBL	7×10^6	100	37	95
THY	7×10^6	9	5	dead
PBL	4×10^7	92	14	79
CLL	17×10^6	96	5	95
THY	3×10^9	95	172	91
CLL	8×10^7	95	38	90
CLL	8×10^7	88	19	71
CLL	6×10^7	72	140	64
THY	1×10^7	62	6	60
THY	1×10^9	87	109	83
THY	7×10^9	dead	48	dead
THY	8×10^8	75	97	57
PBL	5×10^7	100	58	97
THY	4×10^7	91	16	48
THY	2×10^8	89	11	38
CLL	4×10^8	95	47	95
CLL	1×10^8	97	32	89
CLL	3×10^9	100	41	99

PBL: Peripheral Blood Lymphocytes.
THY: Fetal Thymocytes.
CLL: Chronic Lymphatic Leukaemia Lymphocytes.

reproducible results. The recommendations of the IUIS Report (1975) and the WHO-IARC. Workshop (1974) were followed as closely as possible.

12.1 E Rosettes

Technique No.3 Preparation of erythrocyte (E) rosettes on lymphocytes in suspension

1) Sheep erythrocytes, up to six days old, preserved in Alsever's solution, were washed by centrifugation in BSS three times, and finally suspended to 0.5% packed cell volume in BSS.
2) Lymphocytes were prepared at a concentration of 10^6-10^7/ml in 50% fetal calf serum in BSS. (Technique No.1)
3) Equal volumes of lymphocytes and red cells were incubated in screw-capped plastic tubes at 37°C for 15 minutes.
4) After incubation, the tubes were centrifuged at 2 000 rpm for 2 minutes and incubated at 4°C for 18 hours.
5) Half the supernatant was removed by pipette and replaced with 1:60 dilution of fresh 25% glutaraldehyde. The cells were gently resuspended and fixed for 10 minutes at 4°C.
6) Cell spreads were made on glass slides, dried in air and post fixed for 10 minutes in methyl alcohol in a coplin jar.
7) Films were stained by conventional haematoxylin

and eosin and mounted in DPX.

The results (Figure 1) permitted an accurate count of lymphocytes rosetted with two or more erythrocytes. Fetal calf serum was found to improve reproducibility of the test and that overnight incubation at 4°C gave higher rosetting rates (Wybran et al., 1973).

Two variations were introduced into the technique. When testing for rosette inhibition by antisera, a drop was taken before glutaraldehyde fixation, for viability testing by trypan blue exclusion, (Section 11.1.2). Also, instead of staining with haematoxylin and eosin, a histochemical test for non-specific esterase was performed (vide infra) to assess this enzyme activity in rosetted cells (Sher, personal communication).

12.2 EA Rosettes, and EAC Rosettes

Washed sheep red cells were sensitised with an IgG haemolytic antibody to yield the EA reagent. These sensitised cells rosette with macrophages via the receptor for cytophilic antibody (Berken and Benacerraf, 1966). An aliquot of EA reagent was exposed to complement (C) to yield EAC reagent which forms rosettes with both macrophages and B lymphocytes via surface complement receptors. The optimum concentration of antibody and complement were calculated initially by a checkerboard titration which was repeated twice as a check and once when the complement source was changed. Four titrations showed remarkable reproducibility over many months, with results illustrated in Table 3. A haemolysin titre of 1:2 000 and complement dilution of 1:40 is indicated for optimum

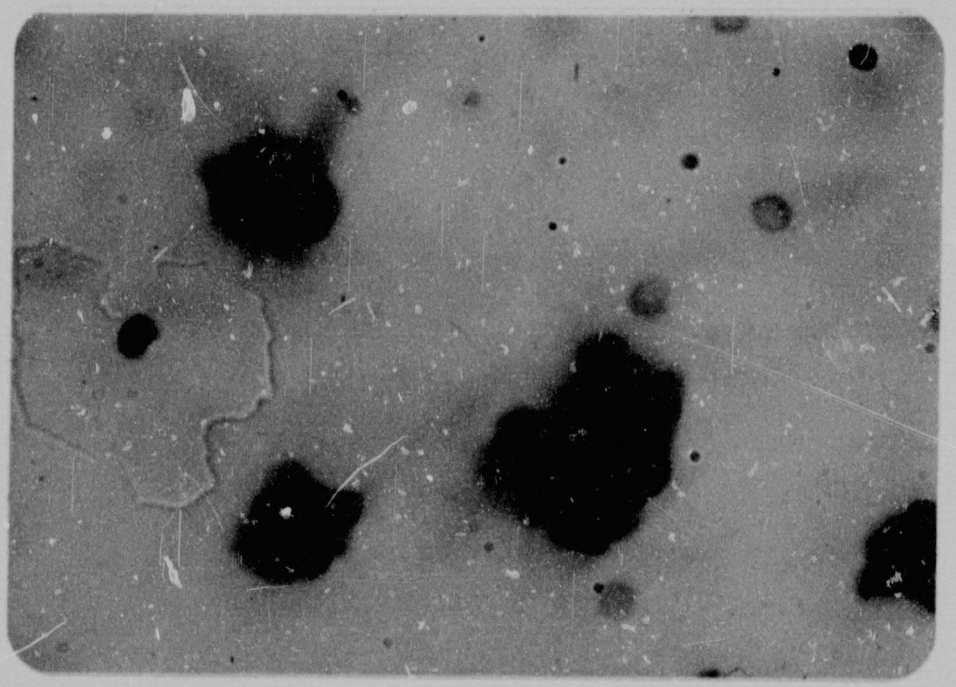


Figure 1 Lymphocyte rosettes. Sheep red cells adhering to thymocytes. Fixed preparation, stained with H & E, x 2000.

Table 3 Sheep red cell haemolysis with antibody and complement dilutions in a checkerboard titration.

	Rabbit anti-sheep haemolysin					
Complement	1:100	1:200	1:400	1:800	1:2000	1:3000
1:20	++++	++++	++++	+++	++	++
1:40	+++	+++	++	+	+	-
1:80	-	-	-	-	-	-

sensitisation.

Technique No.4 Preparation of EA and EAC reagents

1) One volume of 5% washed sheep red cells in BSS (Technique No.3) was added to one volume of rabbit, anti-sheep haemolytic serum (Wellcome Ltd.) diluted to 1:2 000 in BSS.
2) The mixture was incubated in screw capped plastic tubes at 37°C for one hour with intermittent inversion.
3) The preparation was washed three times in BSS by centrifuging and finally resuspended to the original volume of red cells to maintain a 5% suspension.
4) Half the suspension was removed and stored for EA rosette testing.
5) The remainder was added to an equal volume of fresh-frozen human AB group serum diluted 1:40 in BSS, incubated as before (step 2) and washed in BSS (step 3), to prepare a 5% suspension of EAC reagent.
6) Both EA and EAC reagents were stored up to 48 hours at 4°C before being considered unfit for use.

A trace of haemolysis after incubation with complement was desirable as confirmation that maximum sensitisation had taken place. Control material always included cells treated with complement, without sensitising antibody. Guinea pig serum as a source of complement was examined, proving to be

equally successful at a much higher dilution. However human AB serum was more readily available from the Johannesburg Blood Transfusion Service on request.

Technique No.5 Preparation of EA and EAC rosettes on lymphocyte and macrophage suspensions

1) EA and EAC reagents were diluted from 5% to 0.5% suspension in BSS before use.
2) Equal volumes of rosetting reagents and lymphocyte suspensions (10^6-10^7 cells/ml in BSS) were mixed in screw capped plastic tubes.
3) EA reagent and lymphocytes were incubated for 30 minutes at 37°C and centrifuged at 90 g for 10 minutes.
4) EAC reagent and lymphocytes were centrifuged for 10 minutes at 90 g and the tubes incubated at 37°C for 15 minutes.
5) Both preparations were treated as in Technique No.3 (step 5) to produce stained preparations. (Figure 2)

It was confirmed that EA and EAC rosettes were very sturdy requiring no special additives to enhance or retain integrity.

12.3 Rosette technique for sections

The distribution of macrophages and B cells in frozen sections of normal lymphoid tissue was established by applying EA and EAC rosette techniques.

Three normal lymph nodes were kindly made available by Dr. R. Botha of the renal transplant team of the Department

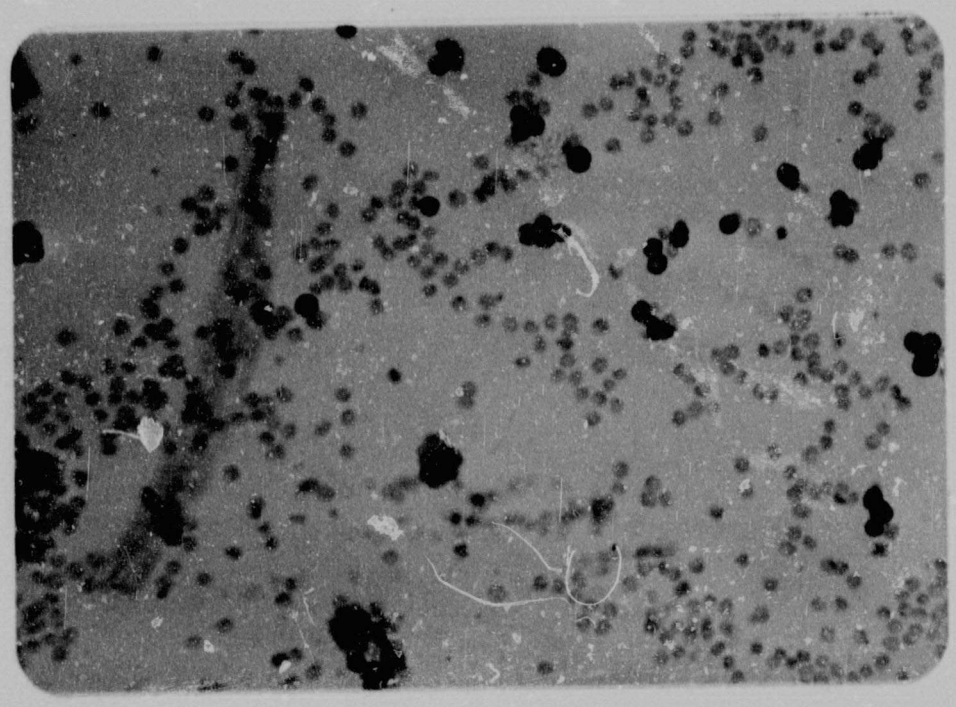


Figure 2 EAC rosettes by peripheral blood lymphocytes.
H & E x 600

of Urology of Johannesburg Hospital. Normal human thymus was obtained from therapeutic abortions as outlined in section 11.3. These were snap frozen in liquid nitrogen and stored therein until used. Sections were cut at 4 μm on an American Optical Co. cryostat for either immediate use, or for storage at -70°C protected by paper tissue and foil. Techniques for EA and EAC adherence to sections were identical, as follows.

Technique No.6 EA and EAC adherence to unfixed frozen sections.

1) The sections were rinsed in physiological saline, buffered to pH 7.4 with 0.6M phosphate buffer (PBS).
2) EA and EAC reagents (Technique No.4), as 5% suspensions, were poured onto the slides, with sections uppermost, in moist chambers.
3) The chambers were incubated at 37°C for 30 minutes.
4) The slides, with sections down, were supported at the ends in a shallow rack made of 1.5 mm perspex, and a gentle stream of PBS passed beneath.
5) When the glass was free of adhered red cells, the sections were fixed in 10% formalin in saline, buffered to pH 6.8, for 15 minutes.
6) After fixation, the sections were stained by conventional H&E and mounted in DPX.
7) To present the illustration in Figure 3, sections with their adhering red cell indicators were illuminated by dark ground microscopy, photographed on colour reversal film, and printed in monochrome

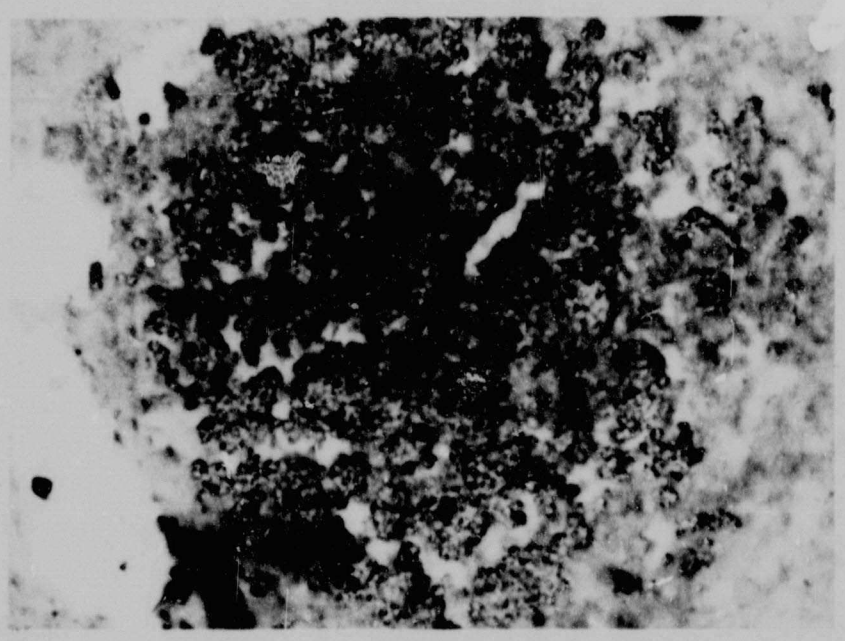


Figure 3 EAC rosettes applied to human lymph node. Dark ground microscopy, reverse printed. Red cells are shown adhering to a follicle, x 720.

as a negative image.

There is some controversy in the literature on whether E rosettes will form on tissue sections (Silvier- et al., 1972; Husby et al., 1975; Shevach et al., 1974; Du et al., 1970). Jaffe et al., (1974) state that E rosettes for.. only on living T cell suspensions and this was the finding during this work. T cell distributions therefore cannot be directly measured by erythrocyte adherence methods. Nevertheless, E rosette reagent (untreated sheep red cells) was always included as a control in rosette testing on sections.

12.4 Surface Immunoglobulin (SIg)

The detection of IgG, IgM and IgA on the surface of lymphocytes was used as a supplementary criterion of B cell identification. (Raff, 1970, Papamichail et al., 1971). No attempt was made to be too critical in procedural design for what could be a most complicated technique.

Technique No.7 Examination of lymphocyte suspensions for surface-bound immunoglobulin (SIg).

1) A commercial preparation containing antibodies to human IgG, IgM and IgA, conjugated with fluorescein isothiocyanate (DAKO) was diluted 1:20 with PBS.
2) Lymphocytes were suspended to 10^6-10^7 cells/ml in PBS after washing (Technique No.1).
3) Equal volumes of cell suspensions and antibody dilutions were mixed in screw capped plastic vials and incubated at $4^{\circ}C$ for one hour.
4) Cells were washed in PBS three times by centrifugation.

5) A drop of washed suspension was mounted under a coverslip, sealed with nail varnish, and examined in a fluorescent microscope (vide infra).

6) Two hundred cells were examined to count those showing granular fluorescence on the membrane.

The results were noted as percent positive for SIg. No attempt was made to prevent "capping" which occurred occasionally nor was any serious attempt made to differentiate positive reactions into individual Ig classes. (Frøland and Natvig, 1972; Fu et al., 1974). It was established that there was good reproducibility of results over a broad range of antiserum dilutions from 1:5 to 1:160. An arbitrary dilution of 1:20 in PBS was chosen, but even at this low dilution the intensity of fluorescence was only just bright enough to permit adequate photograph exposure before quenching (Figure 4).

12.5 Non-Specific Esterases

The references quoted in the introduction were investigated to assess the value of non-specific esterase activity in lymphocyte sub-typing. Meuller's recommendations were applied to normal human lymph node, and dried spreads of cell suspensions. Tissues were fixed overnight in formol-sucrose buffered to pH 6.8, stored in gum sucrose at 4°C, and sectioned on the cryostat. Cell films were dried, fixed in cold Baker's formol-calcium for 10 minutes and washed for 20 minutes in distilled water.

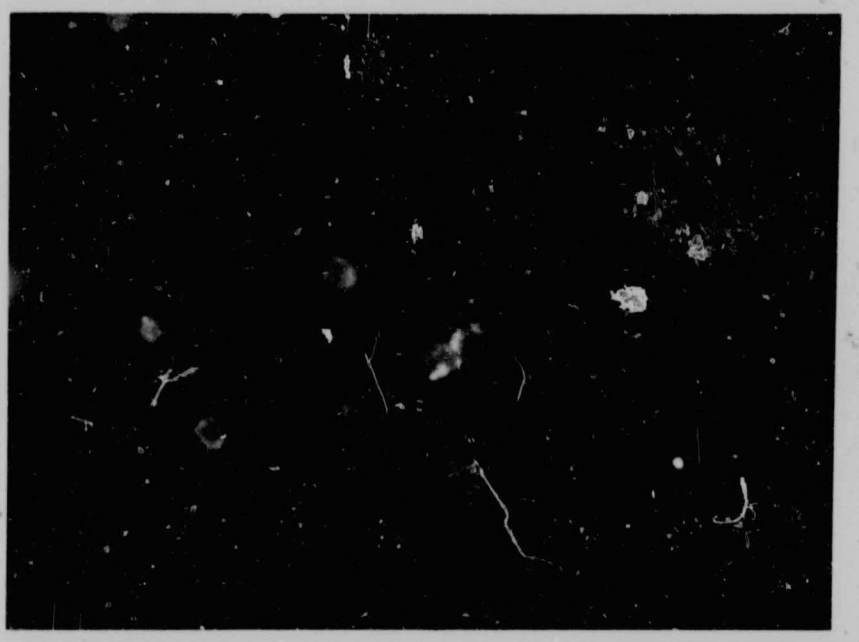


Figure 4 Surface immunoglobulin on lymphocytes. Membrane-bound granules stained by fluorescein-conjugated anti-human globulin, x 1600.

Technique No.8 The demonstration of non-specific esterase activity of mononuclear cells.

1) The freshly prepared substrate consisted of:

α-naphthyl acetate (Light & Co. Ltd.)	10.0 mg
acetone	0.4 ml
phosphate buffer at pH 5.0	40.0 ml
hexazotised pararosanilin	2 4 ml

2) Films and sections were exposed to this substrate in a moist chamber at ambient temperature for between 10 minutes and 2 hours.

3) After rinsing in distilled water, the preparations were counterstained with haematoxylin and mounted in DPX.

In view of the report by Lam and Yam (1973), some alternative substrates were tried, in particular the substituted naphthols. These were synthesized in the laboratory according to Pearse (1972) as follows.

Technique No.9 Synthesis of acetates of substituted naphthols.

1) 5.0 g of naphthol salt was placed in a boiling flask.

2) 10.0 ml of pyridine and 20.0 ml of acetic anhydride were added to the flask in a fume cupboard and a condenser connected.

3) The mixture was boiled under reflux for one hour.

4) At the end of this period the mixture was poured into a large volume of cold distilled

water, when a brown viscous precipitate occurred.

5) The precipitate was collected and dried in a watchglass at 37°C. When dry it was dissolved in excess absolute ethyl alcohol.

6) The solution was shaken with a knifepoint of activated charcoal for 5 minutes and filtered.

7) The alcohol was allowed to evaporate overnight and the purplish crystals of naphthol acetate were recovered for storage.

Thus the acetates of naphthol AS-MX, AS-D, AS-TR and AS-CL (Pfister) were prepared and used as substrates in the above technique at pH 5.0 and 6.0 on human lymph node and guinea pig kidney. None of them showed any greater specificity for T cells, in fact the AS-MX and AS-D gave very weak reactions, so confirming the variable reactions of lymphocytes reported by Lam and Yam. The technique gave results on films made from E rosette preparations (Section 12.1; Sher, personal communication) when it was found that not all thymocytes exhibit esterase activity (Figure 5). Furthermore, macrophages and neutrophils showed marked reactions with most substrates, so their presence in lymph nodes, particularly the marginal and medullary sinuses (Figure 6) made interpretation of cell distributions almost impossible. In view of these findings, no confidence could be placed in non-specific esterase as a T cell marker.

13 ANTISERA PRODUCTION

There are a number of factors to be taken into consideration

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