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The immuno-histochemical differentiation of human lymphoid cells

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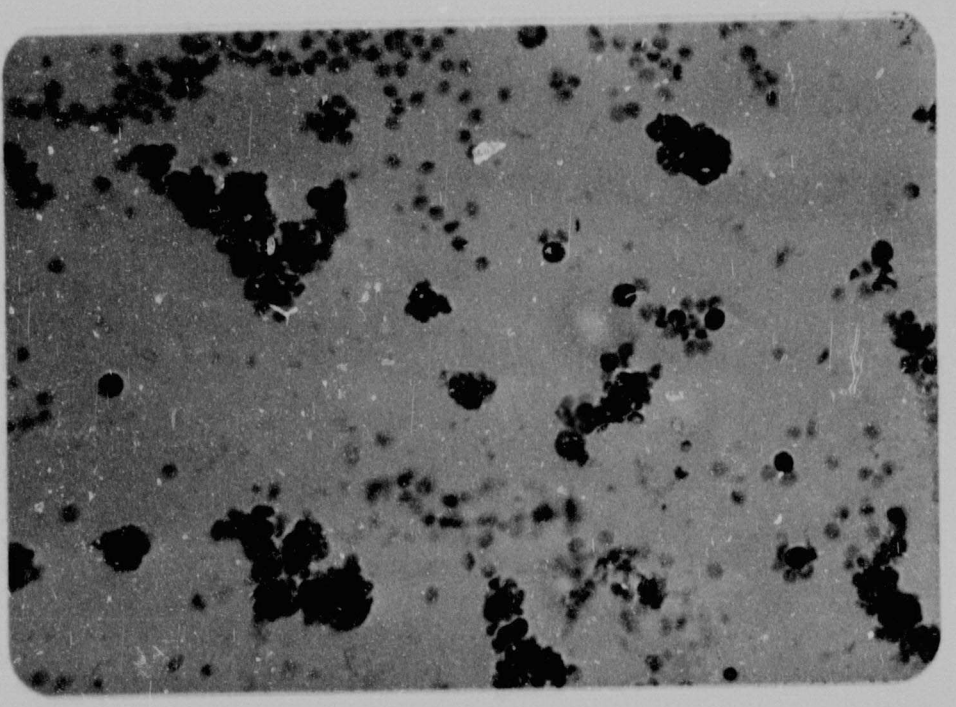


Figure 5 Non-specific esterase activity of E-rosetted thymocytes, x 600.

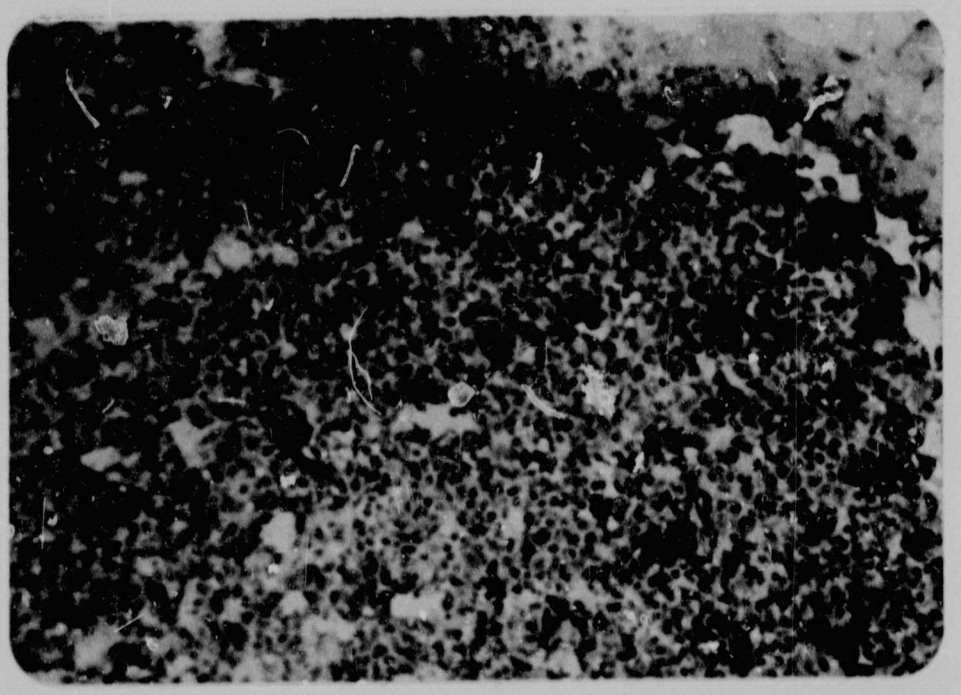


Figure 6 Non-specific esterase activity of human lymph node. Positive macrophage staining is seen in the follicle and subscapsular sinus, x 600.

when attempting to produce specific antisera, some of which are out of the control of the operator. These factors will be discussed in the context of the present study.

13.1 Selection of animals

Although there are reports in human medicine of circulating anti-lymphocyte antibodies (Thomas, 1972) these are of infrequent occurrence and are unlikely to be of value as diagnostic tools. It is necessary therefore to prepare heterologous antisera across the species barrier in suitable laboratory animals. Although all vertebrates have an immune system (Raff, 1973), the goat, horse and rabbit have proved to be most successful (James, 1969). For experimental purposes therefore, the rabbit is the most convenient choice. Age, sex and breed have no significant bearing on suitability for immune responsiveness, rather this is a function of individual variability. This variable may be controlled by using two or three animals for each antiserum. Healthy animals are of course, mandatory, so rabbits for this work were obtained only from reputable dealers or animal houses, quarantined for four days before use, and maintained on commercial pellets, greens daily and water ad. lib.

13.2 Preparation of antigen

The selection of antigen type is dependent to a great extent on the anticipated applications of the projected sera. When clinical immunosuppression is an objective, antisera are usually prepared from ruptured lymphocytes, thus yielding an effective antibody with low toxicity for the recipient.

The use of membrane antigens gives toxic antisera that may nevertheless be suitable for use in laboratory diagnostic techniques. As such was the case here, death and rupture of lymphocytes used for immunisation purposes were avoided as far as possible. This ensured that antibodies were directed only against surface antigens (Woiwod, et al., 1970).

Viable lymphocytes, fresh or from a frozen bank were washed twice in BSS with a final resuspension to at least 10^7 cells/ml. Such suspensions were prepared just before use and never stored. Cells were characterised for the surface markers discussed above to ensure that each inoculum was as pure as possible for the relative antigens.

13.3 Immunisation techniques

In designing an experiment to raise antisera to foreign antigen, one has two broad choices; a short duration course with high antigen concentration, as opposed to extended schedules with adjuvant, but lower doses. Immunosuppression and toxicity are not factors to be considered in preparing diagnostic antisera, so the former method with its assurance of greater specificity was chosen, particularly as it has yielded excellent results in previous experience. (Levey and Medawar, 1966; Gozzo et al., 1971).

Technique No.10 Inoculation of immunising antigen.

1) 1 ml of antigen containing more than 10^7 lymphocytes/ml in BSS were drawn into a tuberculin syringe fitted with a 16 mm, 26 SWG needle.
2) A rabbit's ear was swabbed with 70% alcohol over

the marginal vein and slight pressure exerted at the base of the ear to distend the vein.

3) At venesection, after ensuring that blood could be withdrawn, pressure was released and the contents of the syringe injected slowly.

4) Little difficulty was experienced with post-injection haemorrhage.

Fourteen days later the inoculation was repeated, usually with a different source of cells, into the other ear. Six days later 25 ml of blood was bled from an ear vein for pilot studies.

Technique No.11 Blood collection by venepuncture.

1) The ear over the marginal vein was swabbed with alcohol and xylene to encourage swelling of the vein. A thin film of petroleum jelly was smeared over the area to the edge of the ear.

2) A light longitudinal incision was made into the vein with a sterile scalpel blade and the blood allowed to flow into a plain sterile bottle.

3) At the end of the bleeding, pressure was applied over the wound until bleeding stopped.

4) After coagulation, at 37°C for an hour, the serum was collected by centrifuging and subjected to simple tests for cytotoxicity and rosette inhibition against homologous cells. (Section 11.1.2 and 14.2 vide infra)

Antibody activity was detected in all but one serum and the animals were exsanguinated the next day.

Technique No.12 Blood collection by cardiac puncture.

1) The rabbit was placed under deep anaesthesia by a 1.0 ml, intravenous dose of Nembutal.
2) An 18 SWG needle attached to a 50 ml syringe was inserted between 3rd and 4th intercostal space with the animal on its back. When cardiac movement was felt, a sharp jab placed the needle inside the heart and a free flow of blood was obtained. Between 80-100 ml of blood was collected in two changes of the syringe, before the animal died of anoxia.
3) Clot was separated at 37°C for one hour and clear serum recovered after centrifugation.
4) The serum was inactivated at 58°C for 30 minutes to destroy complement and stored at -20°C until required further.

The rabbit yielding inactive serum was subjected to a further identical course of inoculations starting a week later. Details of the protocols for immunisation of each rabbit are given in Table 4 together with characterisation of the cell suspensions used as antigens.

13.4 Separation of Globulin fractions

To avoid non-specific complications of non-immune serum protein fractions and to save expensive fluorochrome it is usual to separate globulins from antisera, thus allowing greater control in subsequent procedures. Ion exchange chromatography and alcohol precipitation both give a very pure preparation of immunoglobulins, but at this stage such purity

Table 4 Immunisation schedules, doses and antigen characterisation.

Rabbit	Dose	Cells	E rosettes	EAC rosettes	SIg	Cells ml	Viability	Serum code
1	1	PBL	-	12%	-	$4x10^8$	89%	T 1
	2	PBL	-	11%	-	$4x10^7$	75%	
2	1	THY	72%	-	-	$5x10^6$	76%	T 2
	2	THY	85%	-	-	$3x10^7$	96%	
3	1	CLL	15%	25%	72%	$14x10^6$	83%	B 1
	2	CLL	3%	14%	80%	$11x10^7$	71%	
4	1	THY	50%	-	-	$3x10^6$	47%	T 3
	2	THY	98%	-	-	$5x10^7$	95%	
5	1	THY		-	-	$3x10^6$	100%	T 4
	2	THY		-	-	$4x10^7$	93%	
	3	THY	nil	-	-	$2x10^6$	nil	
	4	THY	-	-	-	$7x10^6$	50%	
6	1	CLL	8%	11%	92%	$1x10^8$	95%	B 2
	2	CLL	-	-	87%	$2x10^9$	80%	
7	1	CLL	10%	10%	85%	$16x10^7$	92%	B 3
	2	CLL	8%	11%	92%	$2x10^9$	80%	
8	1	CLL	9%	10%	87%	$13x10^7$	94%	B 4
	2	CLL	8%	11%	92%	$2x10^9$	80%	

is not required. Precipitation with salts is relatively simple with a high yield of crude globulin, so this technique was used on all sera in this study. Increasing ionic salt concentrations precipitate serum protein fractions in a sequence of fibrinogen, euglobulins, pseudoglobulins and albumen (Edsall, 1947). Thus in the absence of fibrinogen in serum, globulins can be precipitated at approximately 45% concentration of ammonium sulphate with little danger of protein denaturation. The reaction is dependent upon initial protein concentration and temperature, but although pH independent, a pH of 7.0 is usually aimed for.

Technique No.13 Salt fractionation of serum globulins.

1) A saturated aqueous solution of ammonium sulphate was made up and stored at 4°C.
2) A measured volume of chilled serum was placed in a suitable flask on a magnetic stirrer and mixed at a speed that just prevented frothing.
3) An equal volume of cold saturated ammonium sulphate was added drop by drop with a pasteur pipette. Globulins started to be precipitated when about two thirds of the volume of added salt solution had been added.
4) The mixture was incubated at 4°C for 18 hours, followed by centrifuging at 3 500 rpm whilst cold.
5) The supernatant was decanted off and the precipitate redissolved in distilled water at pH 7.5, to finish with the same volume as the original serum of step 2. If haemolysis was present in the

original serum the procedure was repeated from step 3.

6) Globulins were dialysed against PBS until the dialysate was clear of sulphate radicle on testing with saturated aqueous barium chloride. (See Section 16.3.1).

The preparations were finally checked by micro-electrophoretic separation on cellulose acetate where it was shown that although albumen was much reduced, all the globulin fractions were still present. (Figure 7)

14 ASSAY OF ANTIBODY

It was observed earlier (Section 13.3.) that there is little correlation between cytotoxicity, immunosuppression, inhibition of function, fluorescent antibody behaviour or even titre of any serum (Johnson and Holborow, 1973). Nevertheless some parameters of the crude globulin preparations were studied. Full evaluation would only be valid in the test conditions of the direct fluorescent antibody procedure.

14.1 Cytotoxicity

The trypan blue exclusion test for cell viability (Section 11.1.2) was applied to lymphocyte suspensions after exposure to globulin preparations. Significant reduction in viability was attributed to antibody activity. Initial experimentation illustrated the need for complement to develop full cytotoxic potential of antilymphocyte sera, so a 1:40 dilution in PBS of fresh frozen AB serum was incorporated into the system. Cytotoxic indices of the sera against homologous cells were

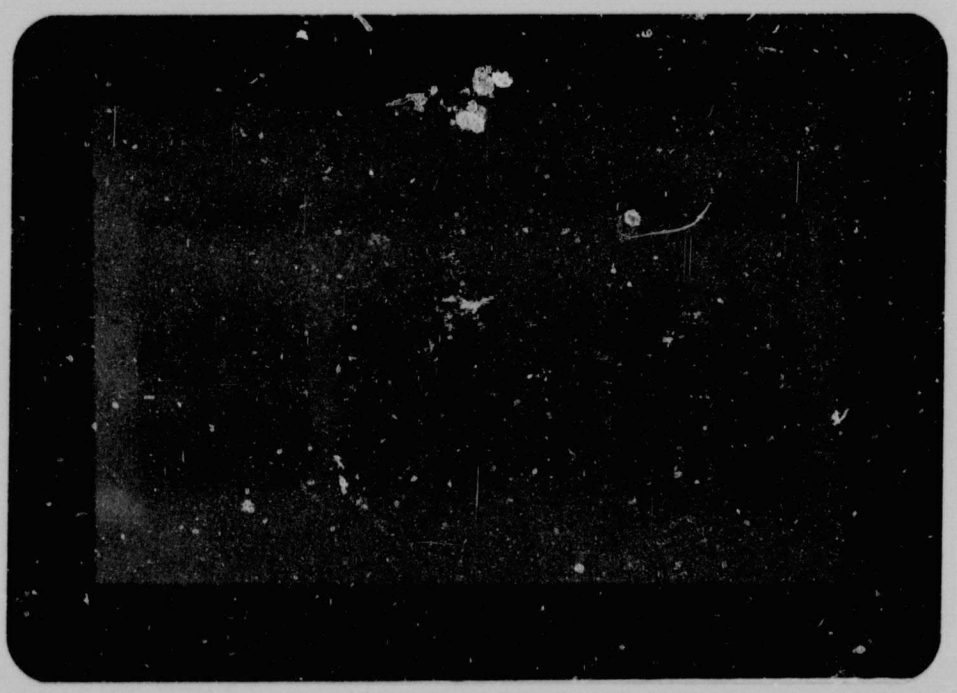


Figure 7 Electrophoretic separation of crude globulins. From left to right, B6, B7, B6, B5, B4, T4, T3. The anode is towards the top of the illustration.

tested at 1:4, 1:8 and 1:16 and 1:32 as follows:

Technique No.14 Measurement of cytotoxic function of antisera

1) Lymphocyte substrate suspensions were adjusted to approximately 10^7 cells/ml; assessed for characterisation by E and EAC rosettes and SIg, with the baseline viability tested by trypan blue exclusion (Techniques 3 and 5).
2) 5.0 ml screw capped plastic tubes were labelled and set up to receive the following reagents;

   Serum dilutions.....3 drops

   Cell suspensions.....3 drops

   1:40 human serum.....1 drop

   The importance of control was never underestimated with most variables accounted for whilst retaining a 7 drop final volume by PBS substitution.

   a) Cells alone to allow for viability deterioration during the test (not significant).

   b) Complement and cells to check the effect of AB serum (never more than 2% decrease in viability).

   c) Normal rabbit serum instead of antiserum; no significant effects were noted with the single serum sample used throughout.

3) All tubes were incubated at 37°C for 30 minutes.
4) Without washing, the contents of each tube was tested by trypan blue staining and examination in a haematological counting chamber.
5) The cytotoxic index was calculated from the

following expression

$$\text{cytotoxic index} = \frac{\%\text{ killed by antiserum} - \%\text{ killed by normal serum}}{100 - \%\text{ killed by normal serum}}$$

It was noted that n· ·toxic sera exhibited strong, complement dependent aggl· ·on of homologous cells that in some cases extended beyond 1:32 before a plateau was reached. Statistical evaluation of the test was done in order to assess the significance of control variables and the effects of serum cytotoxicity. Fifteen identical tubes were set up as described above, using a non-cytotoxic serum, on homologous cell suspensions with baseline viability of 90%. The range of dead cells was counted between 4 and 13%; the mean $\bar{X}$, was 9.1% with a standard deviation of 2.7. The test therefore is surprisingly sensitive, providing there is good procedural standardisation.

14.2 Rosette inhibition

Inhibition of function was tested for in terms of interference with the formation of E rosettes by T cells and EAC rosettes by B cells (Wortis et al., 1973,). The significance of complement was not investigated, as lymphocyte suspensions were made up in fetal calf serum that enhanced E rosette stability but also contributed complement to the system. Lymphocytes were not characterised before the test as this data would become available inherently in the controls.

Technique No.15 Measurement of the inhibition, by antisera, of rosette formation by lymphocytes.

1) Lymphocyte suspensions of 10^6-10^7 cells/ml were

prepared in 50% fetal calf serum in BSS (Technique No.1).

2) Antisera were used in dilutions in BSS from 1:2 to 1:32.

3) Screw capped plastic tubes were set up to receive three drops each of cell suspension and antiserum dilutions in duplicate.

4) The tubes were incubated at 37°C for 30 minutes. Controls included saline and normal rabbit serum substituted for antisera.

5) Cell suspensions, after exposure to antisera were washed twice by centrifuging in BSS. The final wash was decanted completely to leave a pellet of lymphocytes in each tube.

6) Three drops of E or EAC reagents (Techniques 3 and 4) were added to each duplicate pair of tubes and the pellets of cells gently resuspended.

7) Rosetting techniques Nos.3 and 5 were followed exactly and the stained films examined by counting the number of rosettes formed in 100 lymphocytes and comparing this figure with the control suspensions not exposed to antisera.

8) In some experiments, a drop of suspension was taken before glutaraldehyde fixation for testing with trypan blue to study the importance of cell viability in rosette formation.

9) In some E rosette experiments, particularly when thymocytes were used, duplicate films were studied for non-specific esterase activity (Technique No.8)

14.3 Indirect Fluorescent Antibody Tests.

Details of techniques for fluorescent antibody testing are given in section 16.4 in evaluation of conjugates. Globulins were assessed in an indirect test using dilutions in PBS on normal human lymph node for 30 minutes. After washing, antibody activity was visualised with commercial sheep anti-rabbit serum, conjugated with fluorescein isothiocyanate (Wellcome), diluted in 1:4 in PBS, for a further 30 minutes.

15 ABSORPTION OF ANTISERA

To improve specificity of antisera it is necessary to remove reactions that are not specific to the system under study. Apart from naturally occurring inter-species antibodies, immune antibodies may be present in the rabbit serum due either to previous exposure to cross-reacting antigens, or to contamination of primary immunising antigen. Furthermore, some tissue elements are capable of absorbing protein in an entirely non-specific manner (Nairn, 1969). This aspect of antisera production was investigated at length, using the assays in section 14 to evaluate success. As there is a variable loss of antibody activity during absorptions it is necessary to know what are the minimum absorptions compatible with enhancement of specificity without loss of titre. Of the wide choice available the following were chosen for investigation.

15.1 Absorption with human AB cells.

Naturally occurring antibodies to human proteins, present in some rabbit sera, may be removed by using surface antigens

of group AB Rh (D) positive erythrocytes. These were obtained from the Johannesburg Blood Transfusion Service on request, and received up to three weeks old in acid-citrate-dextrose anticoagulant. The cells were washed three times in PBS, ensuring that the buffycoat was removed by suction at each washing to avoid unwanted lymphocyte absorptions. After a preliminary small-scale pilot exposure to make sure that no haemolysis would contaminate the preparation, a volume of serum was added to an equal volume of packed erythrocytes for 2-5 hours, with intermittent inversion, at 4°C. Centrifugation removed the absorbent allowing decantation of clear serum. In most cases, agglutination of the red cells by cold antibodies had taken place.

15.2 Fetal Liver Powder.

A variety of absorbents are recommended for removal of non-specific and connective tissue staining of fluorescent conjugated antisera. Human, rat or guinea pig, kidney and liver may be used, but as fetal material was available (Section 11.3) this was most preferable, particularly as the immature development of the immunologic system would be less likely to interfere with the tenuous anti-lymphocyte antibodies present in the sera to be absorbed. The technique, modified from Nairn (1969), was as follows:

Technique No.16 The preparation of dried liver powder.

1) Deep frozen fetal livers were thawed, minced finely in a domestic mincer, and collected in PBS.
2) The tissue was exposed to five washings in large volumes

(2 litres) of PBS by sedimentation and decantation

3) This was followed by three similar washings in dry acetone to dehydrate and remove fats.

4) Tissue was collected and dried on filter paper for 1 hour at 37°C.

5) The granules were reduced to a powder in a pestle and mortar and dried thoroughly at 37°C overnight.

6) The tissue powder was stored at room temperature until used.

For use, it was felt that it was preferable to allow dilution of the serum rather than have the volume reduced by dry absorbent. To this end, aliquots of powder were placed in tubes in quantities to give approximately 100 mg/ml of serum to be added. The powder was washed once in PBS before addition of serum for incubation at room temperature for 30 minutes. The absorbent was removed by centrifugation.

15.3 Insolubilised serum proteins

B cells by definition carry immunoglobulin molecules bound to their membranes (SIg). This was present on cells used to immunise rabbits (Table 3), and being antigenic could have caused anti-human immunoglobulin antibody production. To separate this activity from antibodies against B cell surface antigens it was necessary to study the effects of absorptions by serum proteins. Some techniques add soluble immunoglobulin to this end, thus blocking antiglobulin activity, but this could not be accepted in the system under study in view of Fc receptors on immunocytes able to bind to such complexes.

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