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The immuno-histochemical differentiation of human lymphoid cells

Hill, Robert Rowland Humphrey

2015-01-27
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Table 6 Cytotoxic indices of anti-CLL cell sera.

	Target cells		
	Thymocytes	CLL cells	PBL
Anti-B 1	0.04	0.00	0.02
Anti-B 2	0.07	0.07	0.05
Anti-B 3	0.17	0.60	0 08
Anti-B 4	0.04	0.05	0.04

population of 11% (B cells). The killing capacity of serum B3 was complement dependent as no effect was observed when human AB serum was omitted from the system (Batchelor 1973). Complement control showed no intrinsic killing potential.

Cytotoxic titres were very poor, as no serum was effective beyond a dilution of 1:64, whereas titres of 1:2048 are reported in the literature (Touraine et al., 1974).

In a complement dependent system such as B3, the source of complement can affect the result to a great extent. As Batchelor (1973) observes, rabbit complement is highly active for human systems whereas human or guinea pig may not be so effective. Figure 8 illustrates the loss of cytotoxicity of serum B3 upon dilution,but there is no defined plateau when titrated against CLL cells. Serum T3 however (Figure 9) showed a plateau of activity against thymocytes to a dilution of 1:8. Cytotoxic specificity is illustrated by the action of reagents on heterologous cells. Although anti-thymocyte sera demonstrated no activity against CLL cells. Anti CLL serum B3 showed an index of 0.35 for thymocytes, which withstood dilution to 1:16.

18 INHIBITION OF FUNCTION.

Anti-thymocyte sera were dramatically effective in inhibiting the formation of E rosettes by thymocytes. This observation however must be taken in consideration of the findings that only viable thymocytes will form rosettes with sheep cells. (Figure 10) There was always correlation between the viability of thymocyte preparations,and the E rosette function (Table 4) there being reciprocal relationship between viable,

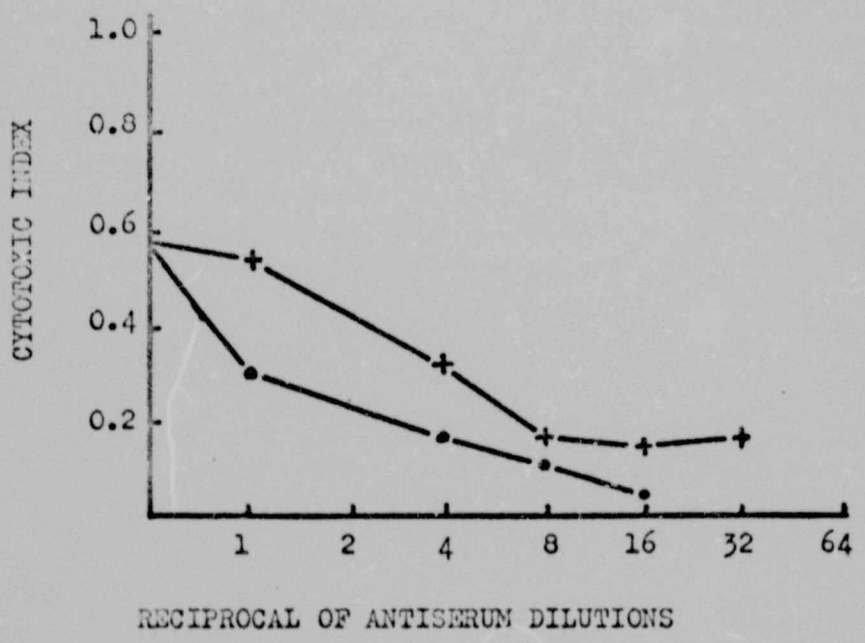


Figure 8 Cytotoxic activity of serum B3 against CLL cells +——+ and Thymocytes ●——●

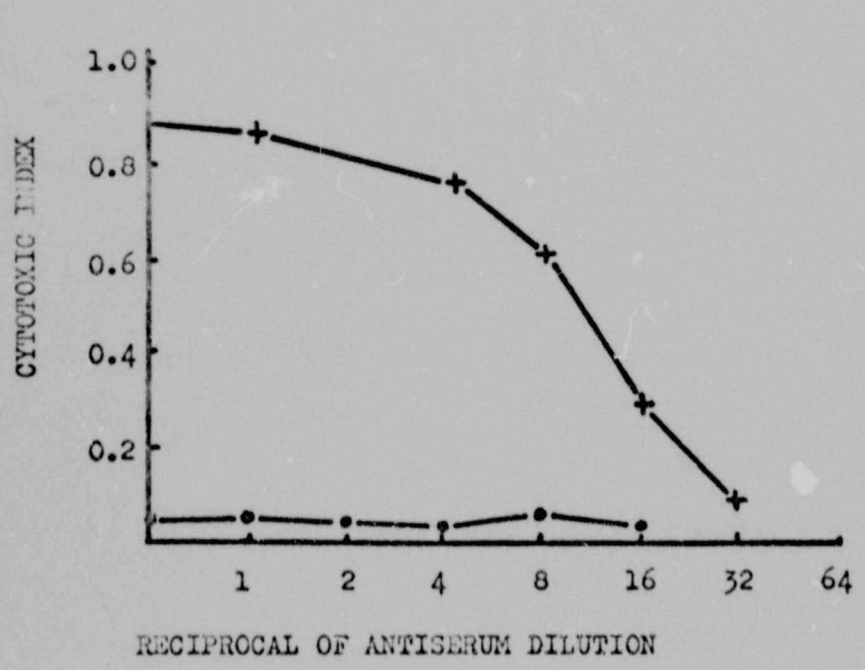


Figure 9 Cytotoxic activity of serum T3 against Thymocytes +——+ and CLL cells ●——●

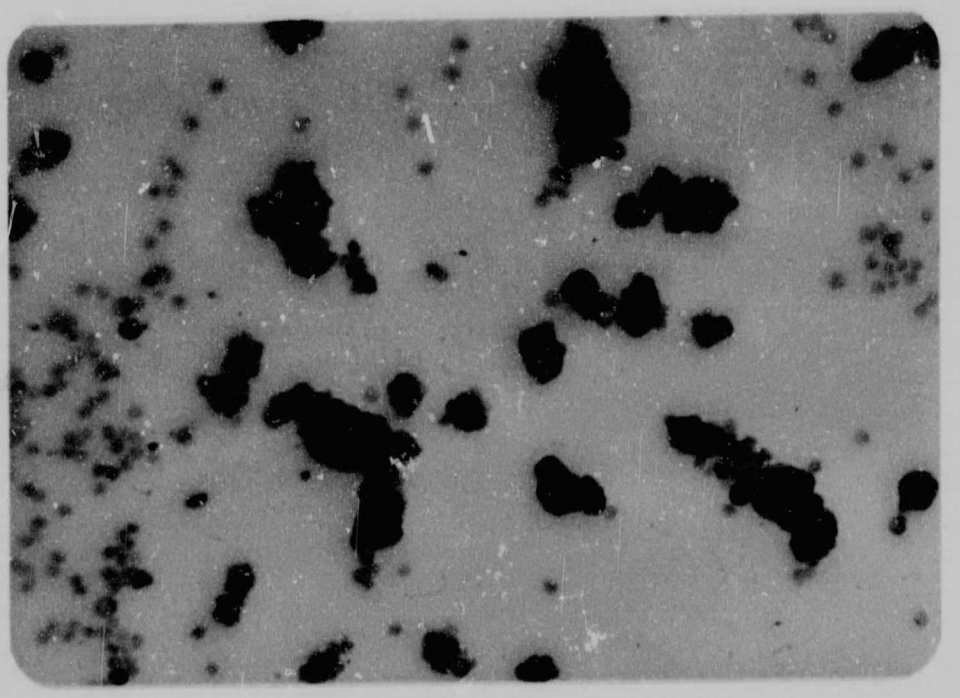


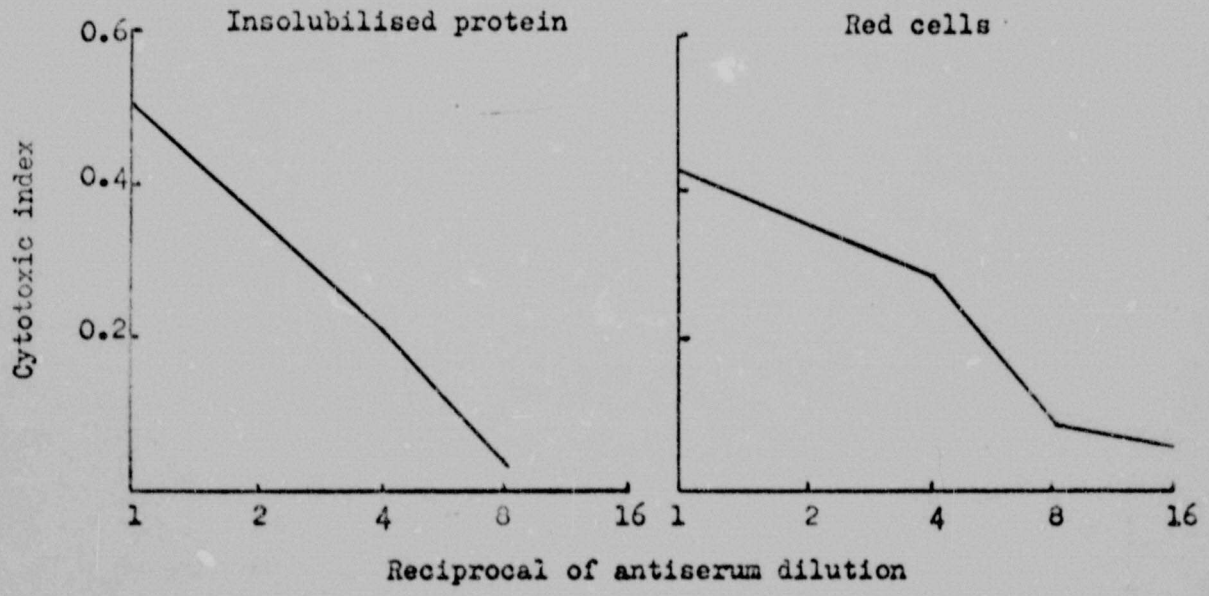
Figure 10 E rosettes on thymocytes, illustrating that almost every cell exhibits this function, H & E x 600.

rosetting cells and non-viable non-rosetting cells. It would be reasonable to assume then, that as the anti-T sera showed high indices of cytotoxicity, the rosette forming capability of target cells would be reduced - the function would be merely another criterion of cytotoxicity as discussed in the previous paragraph. Wortis et al., (1973) found this not to be so, as their anti-T cell sera required complement for cytotoxicity, whereas their rosette inhibition experiments were conducted in the absence of complement. There was a slight discrepancy in our results that tended to confirm the findings of Wortis et al., in that cytotoxic indices of sera at 1:1 dilution were between 0.8 and 0.9 whereas there was 100% inhibition of rosette function. There would appear to approximately 10% of thymocyte populations not affected by anti-thymocyte sera.

Anti-CLL sera did not interfere significantly in the formation of EAC rosettes by B lymphocytes, but this finding was not unexpected. Surface receptors for complement are not dependent upon cell viability, and in a complement system as obtained in the test there is no reason to expect antibody to block such receptors. Anti CLL serum B3 showed a low level of thymocyte kill (Figure 8) and therefore E rosette inhibition.

19 THE EFFECTS OF ABSORBENTS.

Pilot experiments to judge the effects of absorptions on cytotoxicity were conducted to be able to rationalise absorption of bulk sera. Figure 11 shows the effects of four absorptions on serum B3, with fetal liver powder, human AB eryth-



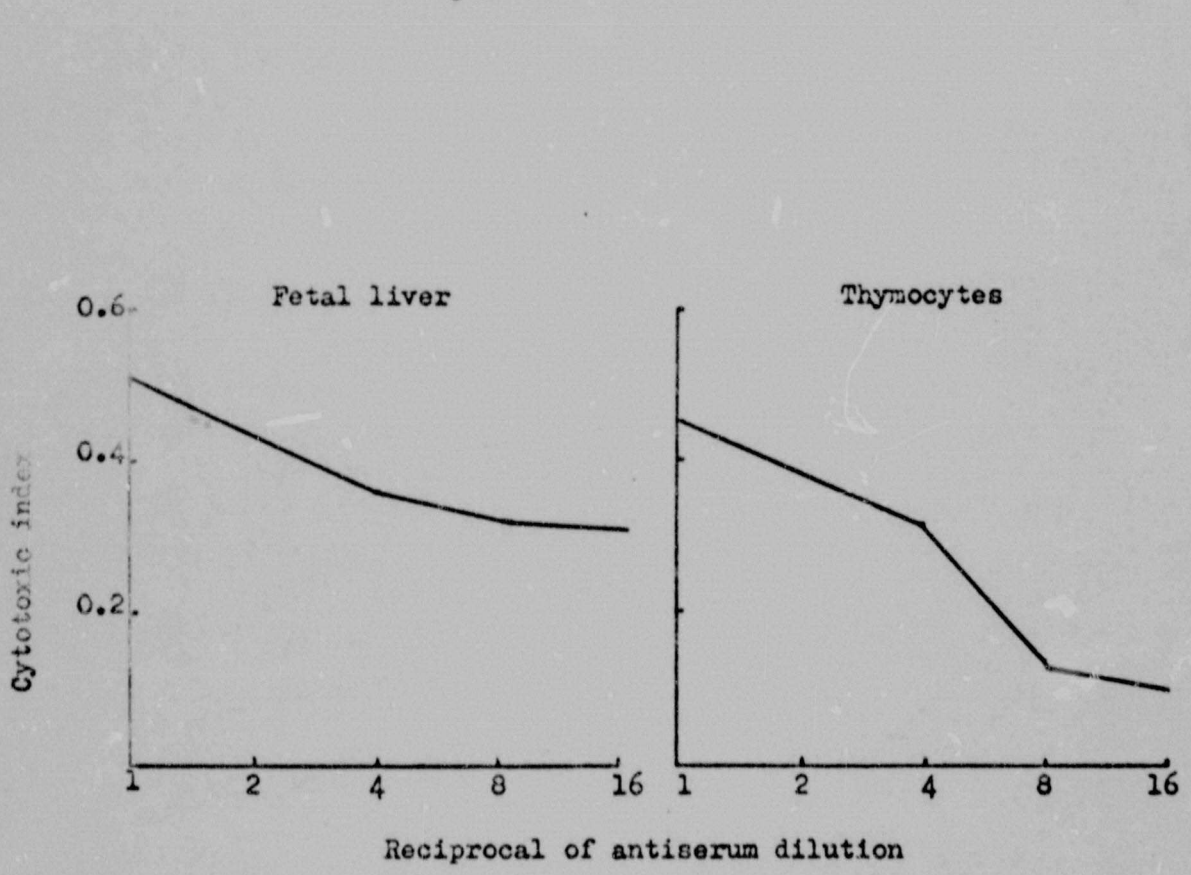


Figure 11 The effects of absorbents on the cytotoxic titres of anti-CLL cell serum (B3) activity against CLL lymphocytes.

rocytes, insolubilised serum proteins and heterologous lymphocytes. The figure shows that fetal liver powder affects cytotoxicity very little, so this treatment was used for all sera to remove non-specific tissue staining by reagents after conjugation with fluorochrome. Thymocytes removed a significant amount of activity of B3 as was to be expected from its non-specific nature. This effect may also be due, however, to some histocompatibility antigens shared by absorbents and immunising lymphocytes. Although rabbits were inoculated with relatively pure suspensions of lymphocytes, it was still necessary to absorb with heterologous cells before conjugation to ensure specificity. Human AB erythrocytes contributed nothing to specificity but removed significant activity from already weak antisera. Insolubilised serum proteins were only used on anti-CLL sera, reducing the titre of serum B3 to 1:8. The initial suspicion that the anti CLL sera contained antibody against immunoglobulin is confirmed later (Section 22.1).

20 CONJUGATION WITH FLUOROCHROME.

After absorption with heterologous cells, the protein values of the bulk sera was measured by Biuret technique (Section 16.1). The data for conjugation with fluorescein isothiocyanate are given in Table 7, which shows the calculations necessary for judging the amount of fluorochrome necessary for optimal conjugation of each serum. After conjugation the preparations were tested for specificity and titre by the direct fluorescent antibody test on normal human lymph node. The criteria for suitability at this stage, were taken as intensified staining of germinal centres by anti CLL sera,

Table 7 Conjugation data for eight sera.

Serum code	Protein mg/ml	Vol. ml	Total prot. mg	Diluted vol. ml	FITC µg
B1	26	3.2	83	8.3	332
B2	22	1.6	35	3.5	140
B3	28	2.0	56	5.6	224
B4	18	2.0	36	3.6	144
T1	18	1.5	27	2.7	108
T2	16	0.4	7	0.7	28
T3	21	3.5	73	7.3	300
T4	20	3.0	60	6.0	240

and no follicular staining with anti-thymocyte reagents. The following results were obtained.

T1: Heavy non-specific staining to a dilution of 1:80.

T3. Good specific staining of T dependent areas to a titre of 1:40.

T4. Good specific staining to 1:20.

B1. General staining of all areas to a titre of 1:40.

B2. No specific staining but heavy non-specific fluorescence.

B3. Potentially good, but some non-specific staining. to 1:40.

B4. Good staining to a titre of 1:40.

21 PURIFICATION OF CONJUGATES.

21.1 Removal of free fluorochrome and non-specific staining.

Methods for removal of free fluorochrome after conjugation were investigated. Activated charcoal treatment and Sephadex G 50 chromatography were compared with fetal liver absorption, using fluorescence specificity as a criterion. Activated charcoal treatment resulted in a severe loss of reactivity, with complete removal of fluorescent staining at low dilutions. Fothergill and Nairn (1961) report up to 40% loss of protein by charcoal extraction. Sephadex G 50 columns were more successful, as free fluorescein was retained by the columns. Recovery of conjugated protein was effected in little more than that of the original applied volume, with minimal dilution by eluting buffer. Combining Sephadex G 50 chromatography with fetal liver absorption afforded the most effective

control of ionic staining by fluorescein and the affinity for charged conjugated protein molecules of some tissue elements.

21.2 DEAE cellulose, chromatography.

Conjugated protein was separated from DEAE cellulose columns by stepwise elution with three buffers, the effluent being collected in ten, 2 ml volumes, at an elution rate of 18.5 ml/hour. Each fraction was assayed for physico-chemical parameters by U.V. spectrophotometry and re-examined for their behaviour in the fluorescent antibody test.

The fractions from the three buffers showed quite different characteristics; hypotonic buffer eluted optimally conjugated protein; isotonic buffer eluted under-conjugated molecules, and hypertonic buffer eluted over conjugated molecules. Most of the free fluorochrome had been removed by Sephadex chromatography. Sera T1, T2 and B2 eluted completely with step 3 buffer so no fractions with usable specific characteristics were recovered. Figure 12 illustrates the protein and fluorochrome concentrations of the eluted fractions of two of the successful sera. Usually fractions 1, 2 and 3 showed satisfactory staining behaviour when tested on lymph node; they showed optimum F/P ratios of between 3.2 and 6.6. Fractions 4 and 5, being unlabelled antibody with F/P ratios below 2.0 gave weak or no staining. Fractions 6 to 10 were intensely coloured with marked non-specific staining of all tissue elements due to the high F/P ratios between 8.0 and 11.0. Fractions from each serum showing satisfactory F/P ratios or specific staining, were pooled, and concentrated

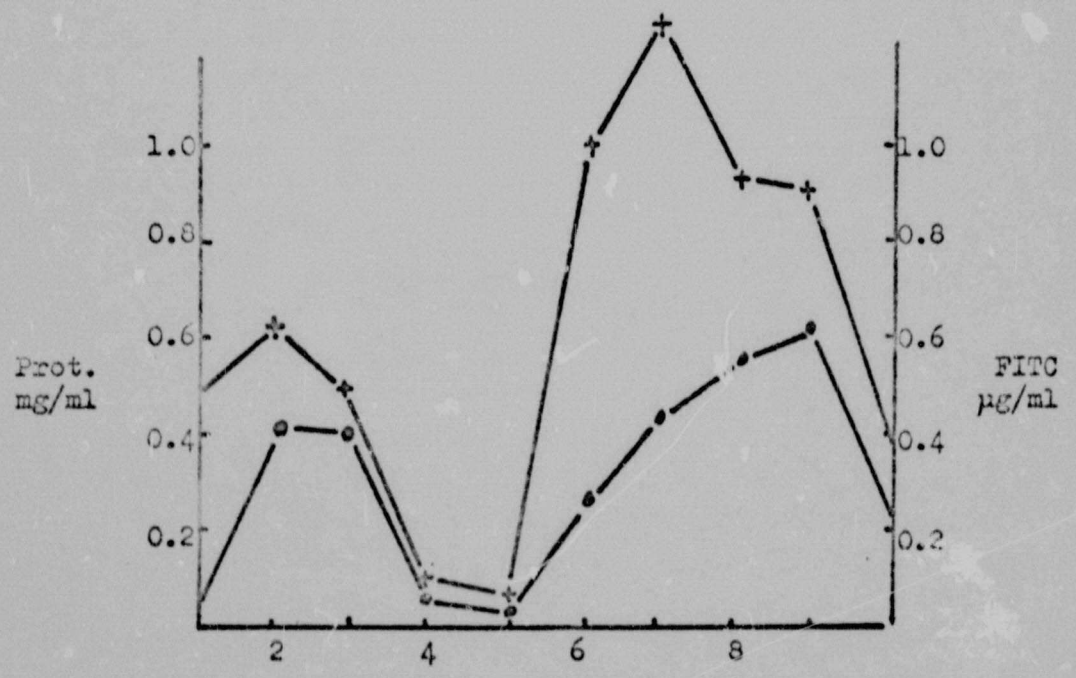


Figure 12 Spectrophotometric analysis of 2.0 ml eluate fractions of antisera after DEAE cellulose chromatography. Protein •——•, Fluorescein isothiocyanate +——+.

by dialysis against 30% polyvinyl pyrrolidone until the volume was reduced to a quarter so as to finish with a protein concentration of approximately 10 mg/ml. The two lots, anti-thymocyte and anti-CLL cell conjugates were dialysed against PBS overnight to restore osmolarity and lyophilised in 1 ml volumes in a commercial freeze drying machine.

Table 8 illustrates the physico-chemical parameters of the pooled, freeze dried conjugates when reconstituted with 1.0 ml of distilled water.

22 FLUORESCENT ANTIBODY BEHAVIOUR AND SPECIFICITY

The antisera described in Table 8 were subjected to critical tests in the direct immunofluorescent procedure on various substrates and controls (Section 16.4).

22.1 Cell suspensions.

The two antisera were mixed with homologous and heterologous lymphocytes in suspension. After incubation, the cells were examined for specific fluorescence and the sera retested in the direct fluorescent antibody test to study the effect of such absorptions. Table 9 shows the results of cross reaction experiments which were seen as the presence or absence of membrane fluorescence illustrated in Figures 13 and 14. The fluorescent emission was almost at the limit of the optical performance of the microscope, due in part, to the limitation of antigen to the cell membranes with a possible paucity of combining sites. This would restrict the energy release of excited fluorochrome to critically low levels, liable to the rapid quenching of the fluorescent image found in

Table 8 Physico-chemical data on pooled conjugates.

Conjugate	OD_{495}	OD_{280}	protein mg/ml	FITC µg/ml	molar F/P.
Anti CLL	0.34	0.62	16.8	88	2.4
Anti thymocyte	0.18	0.28	8.8	48	3.1

Table 9 The results of cross-reaction experiments on lymphocyte suspensions.

	Lymphocyte membrane fluorescence.		
Anti-CLL:	CLL cells	thymocytes	PBL
unabsorbed	93% weak	nil	13% weak
absorbed with CLL cells	79% weak	nil	6% weak
absorbed with thymocytes	82% weak	nil	15% weak
Anti-thymocyte:			
unabsorbed	11%	100%	92%
absorbed with CLL cells	5%	100%	95%
absorbed with thymocytes	8%	7%	6%
Anti-human globulin Serum for SIg	95%	3%	12%

practice.

Although the anti-thymocyte conjugate gave most satisfactory results with adequate specificity for its target cells, the data on the bottom line of Table 9 would indicate that the anti-CLL reagent was in fact detecting surface immunoglobulin and not B cell surface antigens. This is supported by the marked reduction in activity occasioned by absorption with insolubilised serum proteins (Section 19) and the inability of CLL cells to absorb out the lymphocyte membrane staining. The lack of thymocyte staining would therefore lead to false conclusions on anti-CLL cell specificity, as thymocytes do not demonstrate membrane immunoglobulins by standard methods. It was noted again, and illustrated in Figure 13, that anti-thymocyte reagent caused specific agglutination of thymocytes or T cells, in addition to staining their membranes.

22.2 Tissue sections.

Having established the specificity of ant-immunocyte sera, they were examined for their behaviour with T and B cells in tissue sections. Normal lymph node and fetal thymus were prepared as described in Section 12.3, and B cell and macrophage distributions established with EAC and EA sheep cell rosettes. (Technique No.6)

Few EAC rosettes were formed on thymus; those observed being on macrophages as confirmed by EA rosette testing. On lymph node, there was intense adherence of EAC

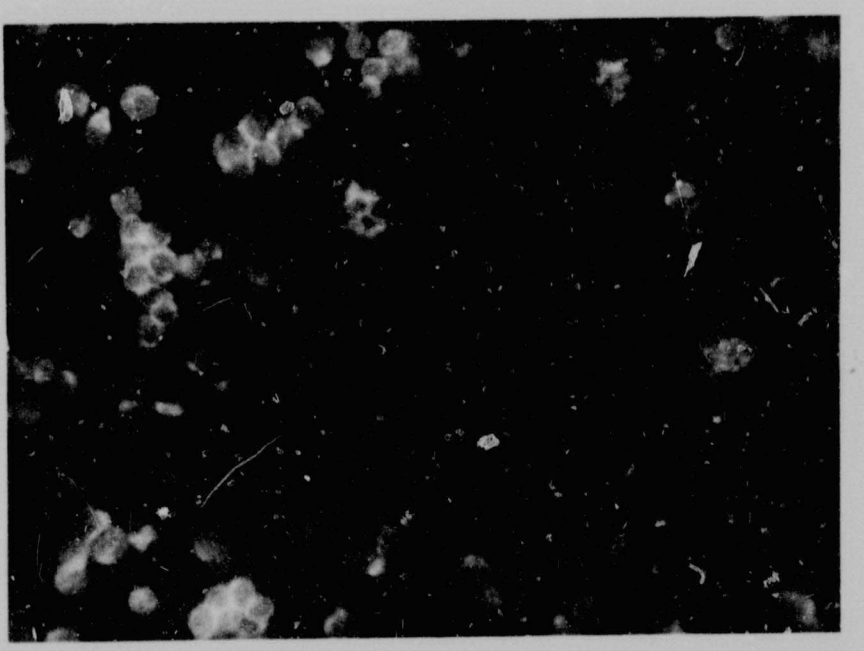


Figure 13 Anti-T cell antiserum activity on thymocyte suspension, x 600.

Author Hill RRH
Name of thesis The Immuno-Histochemical differentiation of human lymphoid cells 1977

PUBLISHER:
University of the Witwatersrand, Johannesburg
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