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Studies on the differentiation of T lymphocytes in sheep

I. RECOGNITION OF A SHEEP T-LYMPHOCYTE DIFFERENTIATION ANTIGEN BY A MONOCLONAL ANTIBODY T-80

M. MIYASAKA, I. HERON*, L. DUDLER, R. N. P. CAHILL†, L. FORNI, T. KNAAK & Z. TRNKA
*Basel Institute for Immunology, Basel, Switzerland, *Institute for Medical Microbiology, Aarhus University, Aarhus, Denmark and †Department of Veterinary Preclinical Sciences, University of Melbourne, Parkville, Victoria, Australia*

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Summary. The results presented in this paper demonstrate that a mouse IgM monoclonal antibody (T-80) recognizes an antigen on cells of the T-lymphocyte lineage of sheep. However, this antibody does not identify all T cells, as 10–20% of thymocytes and some peripheral-blood T cells are negative. T-80$^-$ thymocytes reside in the medulla. The majority of cortical thymocytes are T-80$^+$ and classified as dull cells on the basis of antigen density per cell as measured by flow microfluorometry. In contrast, T-80$^+$ cells in the periphery can be categorized into two populations, i.e., dull cells and bright cells. Suggestive evidence was obtained that bright T-80$^+$ cells are fast recirculating T cells, whereas dull cells are sessile or less easily mobilizable T cells in the periphery.

In foetal environment, over 90% of thymocytes and approximately 5% of spleen cells are T-80$^+$ at 54 days of gestation (gestation period = 150 days), which may indicate that T-cell emigration from the thymus commences well before mid-gestation in sheep.

Abbreviations: FACS, fluorescence-activated cell sorter (-ing); FITC, fluorescein (-ated); Ig, immunoglobulin; PBS, phosphate-buffered saline; sIg$^+$ *or* sIg$^-$ cells, surface immunoglobulin positive or negative cells; T-80$^+$ *or* T-80$^-$ cells, *see note below*; TRITC, rhodamine (-ated).

T-80 is the name of a monoclonal antibody which detects antigen on sheep T cells. It does not indicate molecular weight of antigen. For simplicity we will refer to T-80$^+$ cells as those cells which bind detectable levels of T-80 monoclonal antibody.

Correspondence: Dr Masayuki Miyasaka, Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland.

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INTRODUCTION

The sheep offers good opportunities for studying the physiology of the lymphoid system. Extensive studies have been done on recirculation of lymphocytes in sheep (see review by Hay & Cahill, 1982) and it has been shown in this species that lymphocyte recirculation is established in the foetus (Pearson, Simpson-Morgan & Morris, 1976). It has also been shown that foetal sheep can give immune responses to conventional antigens (Silverstein & Prendergast, 1970; Fahey & Morris, 1978), to allografts (Schinckel & Ferguson, 1953; Silverstein, Prendergast & Kraner, 1964), and to maternal antigens (Miyasaka & McCullagh, 1981, 1982).

However, the paucity of well defined lymphoid-cell surface markers in sheep has often precluded more

detailed definition of many of the interesting phenomena observed in this species.

In this paper, we describe a unique monoclonal antibody (T-80) against thymocytes and some T lymphocytes in sheep. We also present data on the anatomical distribution and ontogeny of cells positive for this antibody.

MATERIALS AND METHODS

Animals

Eight-week-old BALB/c mice were obtained from the Institut für Biologisch-Medizinische Forschung, Füllinsdorf, Switzerland. Sheep of both sexes from commercial flocks of White Alpine and Black Face of various ages were used. Foetuses were obtained from the timed matings of superovulated ewes.

Production of monoclonal antibody

Presumptive sheep T cells were obtained by passing efferent-lymph lymphocytes through a nylon-wool column, as described below, followed by the removal of any remaining surface immunoglobulin positive (sIg^+) cells with the rosetting technique (Parish *et al.*, 1974). An eight-week-old female Balb/c mouse was immunized i.p. with 5×10^6 presumptive T cells at 14-day intervals. Three days after the third immunization, the spleen was obtained and cell fusion was carried out using X63-Ag8 myeloma (a gift from Dr G. Köhler, Basel Institute for Immunology) as described previously (Köhler & Milstein, 1975). A selected hybridoma was cloned twice in soft agar (Kennett, McKearn & Bechtol, 1980) and grown as ascitic tumour in BALB/c mice. The isotype of a monoclonal antibody T-80 was demonstrated to be of IgM class with Ouchterlony analysis.

Surgical procedures

Cannulation of the efferent lymphatic duct of the foetus has been described (Cahill *et al.*, 1979). Cannulation of the efferent lymph ducts of young lambs and adult animals was carried out following the method described by Pederson & Morris (1970). When chronic drainage of lymphocytes was necessary, the lymphatic fistula was maintained for over 30 days, which resulted in the removal of $5–9 \times 10^{10}$ circulating lymphocytes. For the collection of macrophages and granulocytes a fenestrated plastic ball (diameter 4 cm), which carries two silicon-rubber tubings with a three-way valve on each end, was implanted subcutaneously into the lumbosacral fossa of sheep. The tubings were led out through the skin of the flank and anchored on the body. The tissue fluid that accumulated in the plastic ball was obtained by suction from one vent of the ball, while saline was injected into the ball from the other vent. Foetal thymectomy was performed as described previously (Cole & Morris, 1971a).

Isolation of lymphocyte populations

A suspension of single cells was prepared from lymph node, spleen, thymus and Peyer's patches and viable cells were obtained with one-step density gradient separation procedure (Davidson & Parish, 1975). Peripheral-blood lymphocytes were prepared as described before except that 60% Percoll (Pharmacia, Uppsala, Sweden) in Ca^{2+}, Mg^{2+}-free Hanks's medium (Gibco Europe) was used instead of Ficoll–Isopaque (Miyasaka & McCullagh, 1981). Cell separation with nylon-wool column was described (Cahill *et al.*, 1978). Enrichment of sIg^+ B cells was achieved by adherence to anti-Ig-coated plates, according to the technique described by Wysocki & Sato (1978). The viability of the recovered cells was always above 95% and about 93% of the adherent cells were sIg^+.

Isolation of platelets, granulocytes and macrophages

Platelets were collected in the course of separating lymphocytes from peripheral blood. Granulocytes and macrophages were separated from the subcutaneous tissue fluid with 60% Percoll solution. While most of the macrophages remained in a layer above the 60% Percoll solution after the centrifugation at 1200 *g* for 15 min at room temperature, most of the granulocytes were recovered in the pellet. Combined esterase stainings using alpha-naphthyl acetate and naphthol AS-D chloroacetate as substrates (Yam, Li & Crosby, 1971) were used to identify the cell types in each cell fraction.

Immunofluorescence staining

Surface immunoglobulin was identified by incubating 10^7 cells with 50 μl of fluorescein (FITC)-conjugated rabbit anti-sheep immunoglobulin (Nordic 1:10 dilution) for 20 min at 4°. The cells were washed twice with RPMI 1640 and examined. Cells binding T-80 monoclonal antibody were identified by incubating 10^7 cells for 20 min at 4° with 100 μl of 1:50 diluted ascites fluid. The cells were washed twice and then stained with 50 μl of FITC-sheep anti-mouse immunoglobulin (Nordic, 1:10 dilution). Comparable results were obtained with rhodamine-conjugated $F(ab')_2$ goat anti-mouse IgM

(Cappel, Cochranville, PA) as the second layer antibody. The stained cells were assayed by visual examination with a Leitz Orthoplan fluorescence microscope or by flow microfluorometry with a FACS II as described below.

Double staining of the surface immunoglobulins and the molecules detected by T-80 monoclonal antibody was done as follows. Rabbit anti-sheep immunoglobulin was extensively absorbed by Sepharose-coupled mouse immunoglobulins and then labelled with FITC to eliminate cross-reactivity to mouse immunoglobulins in the antiserum. Binding of T-80 antibody was detected by TRITC-conjugated rabbit anti-mouse μ-chain. In this experiment both antisera and conjugates were prepared as described previously (Forni, 1979).

Immunoperoxidase procedure

This was done by using peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO-immunoglobulins A/S, Copenhagen, Denmark) as the second layer antibody (Naiem *et al.*, 1982). 3-Amino-9-ethyl-carbazole (Sigma) was used as the chromogenic substrate (Graham, Lundholm & Karnovsky, 1965).

Flow cytometry analysis and cell separation

Flow microfluorometry and cell sorting were done with a Becton Dickinson FACS II. A Spectra-Physics argon laser was used for excitation with 400 mW output at the 488 nm wavelength. Fluorescence was measured above 520 nm (Ditric Optics long-pass filter) with an EMI 9524 photomultiplier tube at 450 V. All fluorescence measurements were made with the Nuclear Data linear pre-amplifiers at unit gain and subsequently processed in a three decade logarithmic amplifier designed by Dr Hans Koller of F. Hoffmann-La Roche and Co. One hundred thousand cells were analysed for each determination. Data were collected in either 256-channel linear-, or 32×32 dual-parameter distributions. Results are presented either as fluorescence profiles, with the cell frequency on the ordinate and the log fluorescence intensity on the abscissa, or as contour maps, with the log fluorescence intensity on the ordinate and the forward light scatter on the abscissa.

RESULTS

Characterization of T-80 antibody

Table 1 summarizes the results obtained for the binding of T-80 antibody to sheep lymphocytes and other blood cells. Most efferent lymph lymphocytes (74·2%) were T-80$^+$ compared with 22·8% which were sIg$^+$. After efferent-lymph cells had been passed through the nylon-wool column, almost all sIg$^+$ cells were eliminated and 91·5% of the residual cells were T-80$^+$. When B cells were removed from the efferent-lymph lymphocytes by their adherence to rabbit anti-sheep immunoglobulin-coated plastic dishes, 89·1% of the remaining cells were T-80$^+$; 98·1% of the cells that adhered to anti-Ig-coated plastic dishes

Table 1. Binding of T-80 antibody to various cell types of sheep

		% positive*	
		T-80†	Surface Ig‡
I.	Adult efferent-lymph lymphocytes§		
	1. unseparated (n = 17)¶	74·2 ± 7·9	22·8 ± 7·5
	2. nylon-wool purified (n = 4)	91·5 ± 6·8	2·0 ± 1·7
	3. Ig$^+$ cells depleted (n = 11)	89·1 ± 5·4	3·3 ± 1·8
	4. Ig$^+$ cells enriched (n = 10)	1·9 ± 1·0	92·8 ± 4·3
II.	Lamb efferent-lymph lymphocytes**		
	1. normal (n = 3)	59·3 ± 7·2	12·0 ± 4·9
	2. thymectomized (n = 3)	33·7 ± 16·8 (17·0, 33·5, 50·6)††	25·3 ± 21·1
III.	Thymocytes**(n = 5)	80·3 ± 5·6	0·5 ± 0·2
IV.	Sheep red blood cells (n = 2)	0	n.d.‡‡
V.	Platelets (n = 2)	0	n.d.
VI.	Granulocytes (n = 2)	0	n.d.
VII.	Macrophages (n = 2)	0	n.d.

* Expressed as mean percentage ± SD of positive cells for each antibody. Determined by visual immunofluorescence.

† Cells were incubated with 1:50 diluted ascites fluid containing T-80 antibody and subsequently stained with FITC-sheep anti-mouse immunoglobulins (Nordic).

‡ Detected by rabbit anti-sheep immunoglobulins conjugated with FITC (Nordic).

§ Efferent-lymph lymphocytes, red blood cells, platelets, granulocytes and macrophages were obtained from adult animals of 1–2 years of age.

¶ Number of samples examined.

** Obtained from lambs of 1–4 months old.

†† Individual percentages.

‡‡ Not determined.

were T-80$^-$. Most thymocytes (80·3%) were T-80$^+$.

T-80$^+$ cells were markedly reduced in sheep thymectomized *in utero*. In one sheep which was thymectomized at 145 days of gestation (gestation period of sheep = 150 days) and cannulated at 9½ months of age, only 17% of the efferent-lymph lymphocytes were T-80$^+$, compared with 59% of efferent cells from normal animals of the same age. The other two thymectomized lambs showed higher percentages of T-80$^+$ cells (33·5% and 50·6% respectively), but the number of T-80$^+$ cells was only 10–15% of normal as the total lymphocytes count in the lymph was very low.

The two-colour fluorescence experiment showed that T-80 antibody recognized a distinct population of lymphocytes that were sIg$^-$. Efferent-lymph lymphocytes were labelled with FITC-rabbit anti-sheep immunoglobulins absorbed on mouse IgG and then with T-80 antibody, followed with incubation with TRITC-conjugated rabbit anti-mouse μ-chain. No cells showed double staining (Fig. 1).

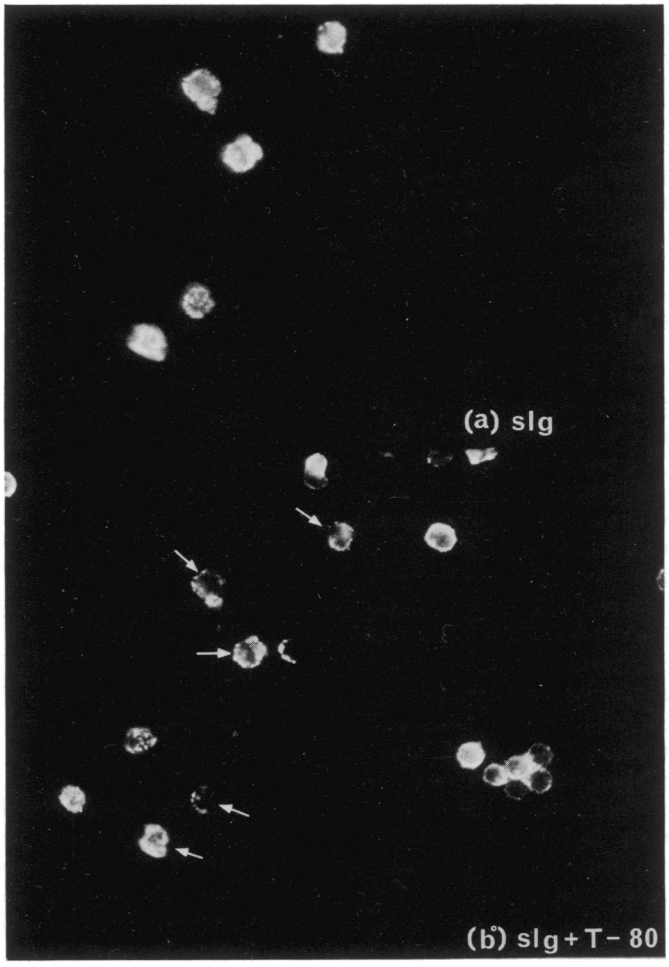

Figure 1. Immunofluorescence of sIg$^+$ and T-80$^+$ cells in the efferent lymph.

Cells were stained with rabbit anti-sheep immunoglobulin conjugated with fluorescein (FITC) and washed three times. Subsequently, the cells were incubated with T-80 antibody followed by incubation with rabbit anti-mouse immunoglobulin conjugated with rhodamine (TRITC). (a) TRITC-stained cells (sIg$^+$ cells). (b) Double exposure was made on the same field to visualize FITC-stained cells (T-80$^+$ cells) and TRITC-stained cells (sIg$^+$ cells). Cells indicated with arrows are sIg$^+$ and the remaining cells are T-80$^+$. No cells showed double stainings.

The distribution of T-80$^+$ cells in tissues

Table 2 summarizes the tissue distribution of T-80$^+$ cells among lymphoid organs, bone marrow and blood. T-80 reacted with 80·3% of thymocytes, 62·2% of prescapular lymph-node cells, 52·9% of mesenteric lymph-node cells, 24·0% of spleen cells and 27·7% of peripheral-blood mononuclear cells. However, T-80 bound only 1·1% of ileal Peyer's patch cells and 12·5% of jejunal Peyer's patch cells. Peyer's patches have been shown to be a major site of B-lymphocyte production in sheep (Gerber, 1979). Ileal Peyer's patches in the sheep consist of numerous primary follicles with scanty interfollicular area, whereas jejunal Peyer's patches contain less follicles and wide interfollicular area (Reynolds, 1976) which is reported to be a T-dependent area in some other species (Parrott, de Sousa & East, 1966).

Flow cytometry analysis

The fluorescence profiles of lymphocytes from various lymphoid organs determined by FACS are shown in Fig. 2. Comparison of the profiles revealed that the thymus and lymph node are rich in cells with intermediate fluorescence intensity or 'dull' T-80$^+$ cells (cells in channel numbers 35 to 100). However, the average fluorescence intensity of dull cells in the thymus is less than that of dull cells in lymph nodes (peak channel difference of 15 channels). The thymus also contained some T-80$^-$ cells which appeared to be located in the thymic medulla. The evidence for medullary location of T-80$^-$ cells is shown in a later section.

Efferent lymph contains a distinct population of bright T-80$^+$ cells (cells in channel number 100 or

Table 2. Tissue distribution of T-80$^+$ and sIg$^+$ cells

Lymphoid organs†	% Positive* T-80	sIg
Thymus (n = 4)‡	80·3±5·6	0·5±0·2
Prescapular lymph node (n = 4)	62·2±6·7	10·2±7·7
Mesenteric lymph node (n = 3)	52·9±15·9	24·1±11·1
Spleen (n = 3)	24·0±4·5	20·5±9·3
Ileal Peyer's patches (n = 6)	1·1±0·4	55·5±21·5
Jejunal Peyer's patches (n = 2)	12·5±5·8	29·5±4·2
Bone marrow (n = 4)	2·0±1·2	2·2±1·1
Peripheral blood (n = 7)	27·7±4·9	29·8±5·2

* Expressed as mean percentage±SD of positive lymphocytes for each antibody. Determined by visual immunofluorescence.

† All organs were obtained from 1–4-month-old lambs. Peripheral blood was obtained from these lambs and also from adult animals of 1–2 years of age. No difference was observed.

‡ Number of samples examined.

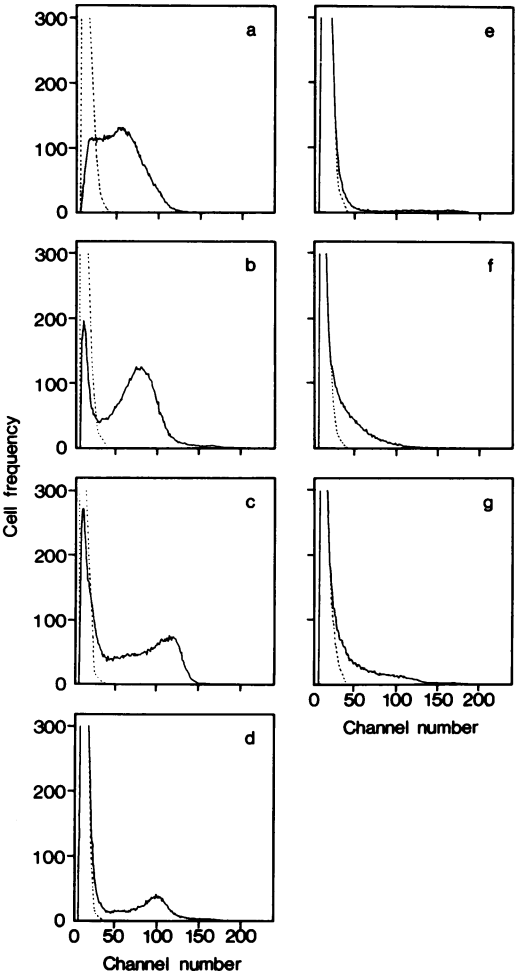

Figure 2. FACS profiles of sheep lymphoid cells labelled with T-80 monoclonal antibody.

After reaction with T-80 antibody, bound antibody was detected by reaction with FITC-conjugated second-step antibody (goat anti-mouse IgM). Stained cells were analysed by FACS. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and linear cell frequency on the ordinate. (——), Profiles of (a) thymocytes, (b) prescapular lymph-node lymphocytes, (c) prescapular efferent-lymph lymphocytes, (d) jejunal Peyer's patch cells, (e) ileal Peyer's patch cells, (f) spleen cells, and (g) peripheral-blood lymphocytes. These profiles are superimposed with a fluorescent profile obtained on unstained efferent-lymph lymphocytes (– – – – –).

more). These bright cells can be observed in jejunal Peyer's patches and to a much lesser extent in other lymphoid organs.

A sharp peak of T-80$^-$ cells observed in all lymphoid organs other than the thymus was judged to be mainly comprised of B cells as this peak disappeared almost completely after the removal of sIg$^+$ cells (profiles not shown). Also ileal Peyer's patches which are rich in B cells but deficient in T cells showed a single peak of negative cells. The spleen and peripheral blood cells showed a similar profile which consists of a sharp peak of negative cells with a broad shoulder on the positive side.

Dual parameter analysis was done to determine whether cells of any particular size possess differential intensity of antibody binding with T-80 monoclonal antibody. Both fluorescence intensity and forward light scatter intensity, which is indicative of cell size, were measured on individual cells. Figure 3 demonstrates a representative example of the contour displays of the dual parameter analyses. It can be seen that there is little or no correlation between cell size and intensity of fluorescence with cells from the thymus and efferent lymph. Both small and large cells were either T-80$^+$ or T-80$^-$. These contour displays demonstrated that the efferent lymph (Fig. 3c) contained a distinctive population of bright T-80$^+$ cells which did not exist in the thymus (Fig. 3a). It is also shown in Figs 3(b) and (d) that jejunal Peyer's patches contained a distinct population of cells which had a similar fluorescence intensity and forward light scatter intensity to a bright T-80$^+$ population of cells in the efferent lymph. This particular population is completely absent in the ileal Peyer's patches.

Ontogeny of T-80$^+$ cells

A study was carried out to investigate the ontogeny of T-80$^+$ cells (Table 3). Most of the thymocytes (92%) and 5·3% of the spleen cells obtained from 54–60-day-old foetuses were T-80$^+$. Cells obtained from the liver were T-80$^-$. A small proportion (0·8%) of spleen cells

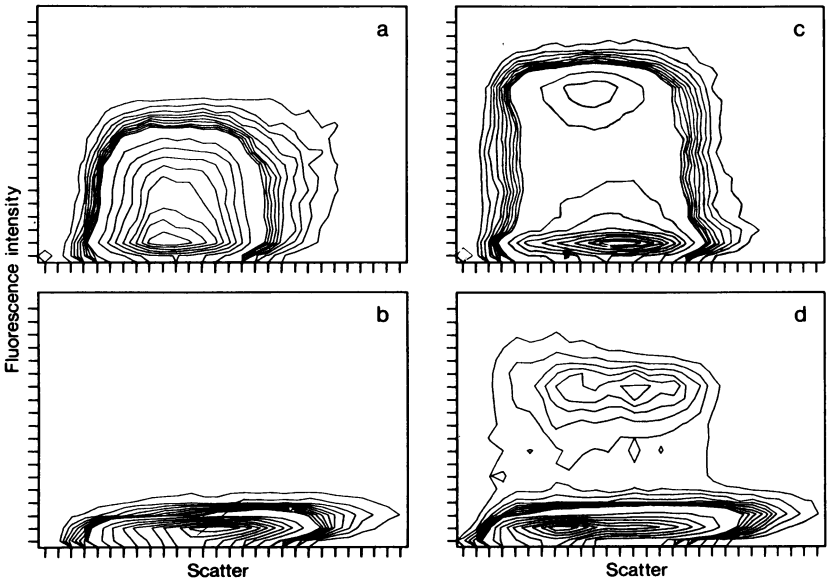

Figure 3. Correlation of cell size, characterized by forward light scatter intensity, with intensity of antibody binding with T-80 monoclonal antibody: contour display.

Abscissa and ordinate represent forward light scatter intensity and logarithmic fluorescence intensity respectively. Both parameters are expressed in arbitrary units. The contours provide a two-dimensional representation of data collected in three-dimensional space, similar to a topographical map. The axis perpendicular to the paper represents the frequency of cells at a given forward light scatter intensity and fluorescence intensity. Individual contour lines show cell frequencies from 1% to 9% and then from 10% to 90% of the peak frequency.

Contour displays of (a) thymocytes, (b) ileal Peyer's patch cells, (c) prescapular efferent-lymph lymphocytes, and (d) jejunal Peyer's patch cells are shown.

Table 3. Tissue distribution of T-80$^+$ and sIg$^+$ cells in foetal lymphoid organs

	% Positive	
	T-80	Surface Ig
54–60-day-old foetuses		
Thymus (n = 6)	90·3±7·8	0
Spleen (n = 3)	5·3±4·6	0·8±1·0
Liver (n = 5)	0	0
136–141-day-old foetuses		
Thymus (n = 5)	77·8±11·4	0·1±0·1
Prescapular lymph node (n = 5)	67·5±17·6	4·3±4·0
Mesenteric lymph node (n = 5)	49·1±23·2	18·5±14·6
Ileal Peyer's patches (n = 3)	3·8±2·6	18·2±13·7
Spleen (n = 3)	28·1±13·2	7·8±2·2
Liver	n.d	n.d.
Peripheral blood (n = 2)	44·9±1·2	2·6±0·6
Efferent lymph (n = 6)	66·6±15·0	4·3±4·0

See footnotes to Table 2.

were sIg$^+$. Lymph nodes and Peyer's patches are very small in foetuses at this stage and were not studied. In older foetuses (136–141 days old), T-80$^+$ cells were found to be the major population in the thymus and in efferent prescapular lymph. The percentage of T-80$^+$ cells in all lymphoid tissues in older foetuses, except the spleen, was very similar to that observed in postnatal and adult animals. The foetal spleen is less lymphoid than that of postnatal animals.

Histological localization of T-80$^+$ cells in lymphoid organs

Frozen sections of the thymus, incubated with T-80 antibody and stained by the immunoperoxidase method, showed that T-80$^+$ cells were the major constituent of the cortex. The medulla was deficient in T-80$^+$ cells. T-80$^+$ cells were most abundant in the white pulp of the spleen, particularly around arterioles. T-80$^+$ cells were present in the mid- or deep cortex of lymph nodes but not in primary follicles and germinal centers. The jejunal Peyer's patches possessed discrete areas of T-80$^+$ cells between the follicles, whereas the ileal Peyer's patches contained only a few T-80$^+$ cells. The majority of cells in the interfollicular areas were strongly T-80$^+$.

Reactivity of anti-T-cell antibodies to brain tissue has been reported in other species (Thiele, Stark & Keesner, 1972; Garson *et al.*, 1982). No T-80$^+$ cells were detected in the frozen sections of the brain except for some weakly stained Purkinje cells in the cerebellum.

DISCUSSION

T-80 monoclonal antibody recognizes a subpopulation of ovine T cells and the following points indicate that there is differential expression of the antigen detected by the T-80 monoclonal antibody. (i) Around 10% or more of thymocytes are T-80$^-$ and the majority of these cells are located in the medulla. (ii) While most lymphocytes in the circulating lymph can be classified in either sIg$^+$ or T-80$^+$ cells, 30–40% of lymphocytes in the peripheral blood are negative for both markers. Preliminary experiments show that these 'null' cells pass through a nylon-wool column as equally well as T-80$^+$ cells, respond by proliferation to alloantigens (Miyasaka & Dudler, unpublished observations), which indicates the presence of T cells with undetectable levels of an antigen recognized by the T-80 monoclonal antibody.

The surface phenotype of lymphocytes may reflect the functional capacity of the migratory properties of the cells (Reinherz & Schlossman, 1980; Swain, 1981). Observations that a bright T-80$^+$ population (measured by FACS) is a characteristic population in the circulating lymph, and that this population tends to be replaced after the chronic drainage by dull cells, which are the major T-cell population in the lymph node, may suggest that there is a correlation between the phenotype of lymphocyte and their migratory properties, i.e. bright T-80$^+$ cells belong to the fast recirculating population, while dull cells are sessile or less easily mobilizable T cells. To the best of our knowledge, a similar monoclonal antibody has not been reported in other species.

The presence of T-80$^+$ cells in the bone marrow needs further clarification. They might be recirculating mature T cells and/or immature haemopoietic cells. The existence of haemopoietic cells bearing T-cell antigens has been reported in some species (Goldschneider *et al.*, 1980; Berman & Basch, 1982; Schrader, Battye & Scollay, 1982).

Only a few attempts have been made to investigate the surface markers on sheep T cells. In one study, it was shown that 70·4% of efferent-lymph lymphocytes and 17·1% of peripheral-blood lymphocytes in adult animals bound sheep erythrocytes in the presence of 14% Ficoll–saline (Outteridge, Fahey & Lee, 1981). Although it was claimed by analogy that these rosette-

forming cells belong to the T-cell lineage, since T cells in other species bind sheep erythrocytes, no evidence has been presented that they are actually T cells. The thymic dependency of this population was not demonstrated. Peanut agglutinin (PNA) has also been claimed to be a T-cell marker in sheep (Fahey, 1980). However, our preliminary experiments revealed that not only T cells but also ileal Peyer's patch cells, the majority of which are T-80$^-$ and sIg$^+$, possess receptors for PNA (Miyasaka & Dudler, unpublished observations). Therefore, PNA does not appear to be qualified for a restricted T-cell marker in sheep.

Whilst there is general agreement that sheep peripheral T cells derive from the thymus, there has been controversy on the function of the thymus in sheep since Cole & Morris (1971a, b, c) demonstrated that foetal thymectomy performed after 62 days of gestation does not affect cellular and humoral immune responses to antigens in sheep after birth. However, we have shown that 92% of the thymocytes and 5% of spleen cells are already T-80$^+$ in 54–60-day-old foetuses (Table 3), which appears to indicate that substantial cell migration from the thymus into the periphery occurs before day 54 of gestation. Thus, it is likely that the thymectomized lambs *in utero* studied by Cole & Morris showed almost normal T-cell responses, because of the proliferation of T cells which had been educated in the thymus and emigrated before thymectomy into the periphery.

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