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The sheep analogue of leucocyte common antigen (LCA)

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Summary. The tissue distribution and immunochemical properties of antigens recognized by two monoclonal antibodies 1.11.32 and 1.28.124 define the sheep analogue of the leucocyte common antigen found in rat, man and mouse. Histological and immunofluorescent studies show that this antigen is found on all lymphocytes, as well as other leucocytes but is absent from non-leucocytic cells. Immunochemical data show that a series of proteins of high molecular weight (190,000–225,000) are recognized, and histological studies show the presence of this antigen on a subpopulation of fetal liver cells as early as Day 27–30 of gestation, and on all fetal thymocytes from Day 40 of gestation.

INTRODUCTION

The long gestation period of 150 days for the sheep makes it an ideal choice to study the ontogeny of differentiation antigens. The development of the fetal lymphoid apparatus of the sheep has been described (Cole & Morris, 1973; Jordan, 1976). Macroscopically, the fetal thymus is first apparent around Day 35 of gestation (Cole & Morris, 1973; Jordan, 1976), while the lymph nodes and spleen appear at 50–60 and 55–60 days, respectively (Cole & Morris, 1973). Lymphocytes are present in the lamina propria of the fetal intestine by Day 120 (Cole & Morris, 1973). Initially, the fetal lymphoid organs appear undifferentiated, with lymphocytes scattered throughout a connective tissue reticulum, but as gestation proceeds these organs acquire an adult appearance (Cole & Morris, 1973; Jordan, 1976).

Two monoclonal antibodies have been produced which, based on tissue distribution and the immunochemical properties of the purified antigen, recognized an analogue of the leucocyte common antigen (LCA) described in the rat (Fabre & Williams, 1977), human (Dalchau, Kirkley & Fabre, 1980) and the Ly5/T200 antigen of the mouse (Scheid *et al.*, 1982). These antigens are expressed only on cells of haemopoietic lineage (Fabre & Williams, 1977; Scheid & Triglia, 1979; Dalchau *et al.*, 1980); however different haemopoietic lineages express different molecular forms and different densities of these antigens. The appearance of LCA in fetal sheep tissues is described.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; HAR, horse $F(ab')_2$ anti-rabbit Ig; LCA, leucocyte common antigen; MW, molecular weight; NP-40, nonidet-P40; PBS, phosphate-buffered saline; PBS/Az, phosphate-buffered saline, 15 mM sodium azide; RAM, rabbit $F(ab')_2$ anti-mouse IgG; RAS, rabbit anti-sheep Ig; SDS, sodium dodecyl sulphate; SDS-PAGE, polyacrylamide gel electrophoresis in SDS; TMRITC, tetramethyl rhodamine isothiocyanate.

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MATERIALS AND METHODS

Animals, cell suspensions and cell lines

BALB/c mice (Walter and Eliza Hall Institute, Mel-

bourne) were used for immunization and ascites production. Polled Dorset or Merino sheep were used as a source of efferent duct lymphocytes (Lascelles & Morris, 1961; Heitman, 1970). Tissues for histology and immunochemistry were obtained either from penned sheep or an abattoir. Peripheral blood lymphocytes were separated from blood using Ficoll-Isopaque density centrifugation. Fetal ages were estimated by measuring crown-rump lengths (cf. Jordan, 1976).

Antibodies

Purified rabbit $F(ab')_2$ anti-mouse IgG (RAM) and horse $F(ab')_2$ anti-rabbit Ig (HAR) were the kind gift of Dr A. F. Williams, Oxford. ^{125}I-labelling of immunogobulins was carried out using the chloramine T method. Rabbit anti-sheep Ig (RAS) was produced as described by Beh & Lascelles (1973). Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TMRITC) conjugation of immunoglobulins was based on the method of Nairn (1976). Peroxidase-labelled rabbit anti-mouse Ig was purchased from DAKO (Copenhagen, Denmark).

Production of monoclonal antibodies

BALB/c mice were immunized four times intraperitoneally at weekly intervals with 1×10^7 sheep efferent lymphactic duct lymphocytes in phosphate-buffered saline (PBS) (pH 7·3). A final intravenous injection of 3×10^7 lymphocytes was given four days prior to fusion. Fusion of splenic lymphocytes from the immunized mouse with p3-NS/1-Ag4-1 (NS-1) cells was carried out as described by Galfre *et al.*, 1977. Positive hybrids were cloned by limiting dilution. Ascites was produced by the inoculation of pristane-primed BALB/c mice with $1–2 \times 10^7$ hybridoma cells.

Glutaraldehyde fixing of cells

Cells were fixed at 1×10^8/ml in 0·125% glutaraldehyde in PBS, 15 mM sodium azide (PBS/Az). The reaction was allowed to proceed on ice for 5 min before being stopped by the addition of 1/10 volume of 10% BSA in PBS/Az. The cells were then washed and stored at −20° in 5% BSA in PBS/Az.

Lymphocytes were fixed to polyvinyl plates using glutaraldehyde and then used in assays following the method of Stocker & Heusser (1979).

Indirect radioimmunoassays

Fresh and glutaraldehyde-fixed cells were used as targets for reacting with hybrid supernatants when assayed for activity. The indirect trace binding assay used was as described by Morris & Williams (1975).

The subclasses of the monoclonal antibodies were determined using a three-step indirect radioimmunoassay with rabbit anti-mouse Ig subclass antibodies (Chemicon International, Los Angeles, CA) as the second antibody and ^{125}I-HAR as the third antibody.

Immunofluorescent staining

The assay used for immunofluorescent staining was basically that used for the indirect radioimmunoassay of cells, except that a fluoresceinated second antibody was used rather than an iodinated one. Freshly prepared cells were used and the assay was carried out at 4°. Following staining the cells were analysed either by a fluorescent activated cell sorter (FACS II, Becton-Dickinson Electronics Lab., Mountain View, CA) or on a Zeiss miroscope equipped with an epifluorescence attachment, selective filters for FITC and TMRITC and a 63Ph oil objective.

Immunohistochemistry

Antigens were localized on sections of paraffin-embedded and frozen tissues using an indirect immunoperoxidase method with diaminobenzidene tetrahydrochloride as the substrate (Barclay, 1981). Prior to paraffin embedding, the tissues were fixed in alcohol (Sainte-Marie, 1962) or in glutaradehyde/paraformaldehyde (Morris & Barber, 1983).

Purification of ascites and antibody coupling to Sepharose 4B

Immunoglobulins were precipitated from ascitic fluid of 1.11.32 inoculated mice, and washed with 40% ammonium sulphate. The immunoglobulins were dialysed against 25 mM NaCl, 25 mM Tris, pH 7·4 at 4° prior to passage through DEAE-52 cellulose (Whatman, Kent) previously equilibrated with the above buffer. The monoclonal activity passed unretarded through the column. Coupling of the antibody at a ratio of 10 mg protein/ml of swollen gel to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) (1.11.32-Seph) was performed according to the manufacturer's instructions.

Radiolabelling of cell surface proteins and immunoprecipitation

Lymphocytes were labelled with ^{125}I using the lactoperoxidase method of Goding (1980). Labelled cells

were lysed using a lysis buffer containing 0·5% (v/v) nonidet P-40 (NP-40) in PBS/Az at 4°.

Immunoprecipitations were performed by adding either 20 μl of 1.11.32-Seph, or 100 μl of 1.28.124 supernatant followed by 20 μl of Protein A-Sepharose. The Sepharose beads were washed three times with lysis buffer prior to being boiled for 5 min in 2·5% sodium dodecyl sulphate (SDS) sample buffer, with and without 1% dithiothreitol (DTT), and then subjected to discontinuous polyacrylamide gel electrophoresis in SDS (SDS-PAGE) (cf. Laemmli, 1970).

Gels were fixed and stained with Coomassie brilliant blue, destained, dried and exposed to XAR-5 X-ray film (Kodak) at −70° with a Cronex fluorescent intensifying screen (Dupont, Wilmington, DL).

RESULTS

Characterization of the monoclonal antibodies

Several hybrid lines were produced which recognize sheep lymphocytes. Two of these, 1.11.32 and 1.28.124, had an identical pattern of antigen recognition. Material eluted from a 1.11.32-Seph column, when bound to PVC and used in an indirect radioimmunoassay, was bound by 1.28.124. Isotyping of the monoclonal antibodies showed that 1.11.32 was IgG1 while 1.28.124 was IgG2a.

Tissue distribution of the antigen detected by 1.11.32 and 1.28.124

Lymphocytes from thymus, spleen, lymph node, efferent lymph, Peyer's patches and peripheral blood were studied using indirect immunofluorescence (Fig. 1). A number of individuals was used for each organ. Although virtually 100% of lymphocytes were positive in each case, the level of antigen expression varied. Within the thymus, there were three peaks of expression, the medullary cells having the most antigen. Macrophages from lung washes were also positive, as were granulocytes. Erythrocytes were negative. Double-labelling studies using TMRITC-RAS IgG showed that the duller cells from 1.11.32 labelling were also sIg+. Both monoclonal antibodies caused agglutination of lymphocytes.

Tissue sections from thymus, lymph node, spleen, bone marrow, small intestine, heart, diaphragm, lung, mammary gland, uterus, ovary, liver, kidney, adrenal gland, brain and salivary gland were examined. In all tissues, only the leucocytic elements reacted positively

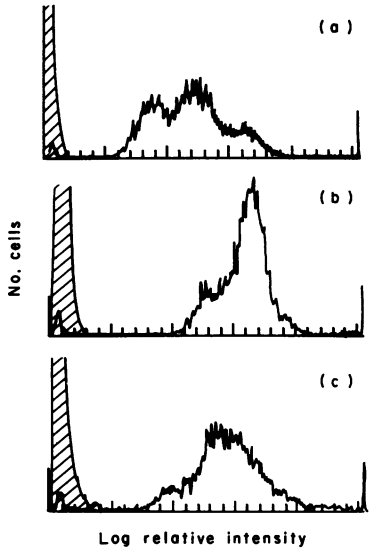

Figure 1. Cytofluorographic analysis of 1.11.32 on teased cell suspensions from (a) thymus, (b) mesenteric lymph node, and (c) spleen. 10,000 cells were counted for each analysis. The shaded portion of each graph shows background staining using a non-specific mouse monoclonal antibody.

(Fig. 2). In the liver, only the Kupffer cells were positive. In the thymus, the medullary cells were more strongly stained than the cortical cells, whilst the cells adjacent to the interlobular connective tissue were the weakest staining. In lymph nodes and spleen, the cells surrounding the follicles were more heavily stained than the follicular cells.

Presence of the antigen in fetal life

The earliest embryos studied were at Day 27–30 of gestation. At this stage of development, most of the cells positive for this antigen were found in fetal liver (Fig. 3). These cells appeared haemopoietic in morphology. Isolated positive cells were seen elsewhere. Virtually all lymphocytes expressed the antigen in Day 40 fetal thymus (Fig. 3). At this stage, there was no differentiation into cortex and medulla. By Day 50, the cortex was distinct from the medulla and there was very little change in the pattern of expression of this antigen in the developing thymus throughout the remainder of gestation. All lymphocytes expressed this antigen in fetal spleen and lymph node from Day 60–65 and Day 55–60, respectively (the earliest time they were examined), although at this stage these organs were relatively undifferentiated. This antigen was also present on isolated cells found in fetal lung at

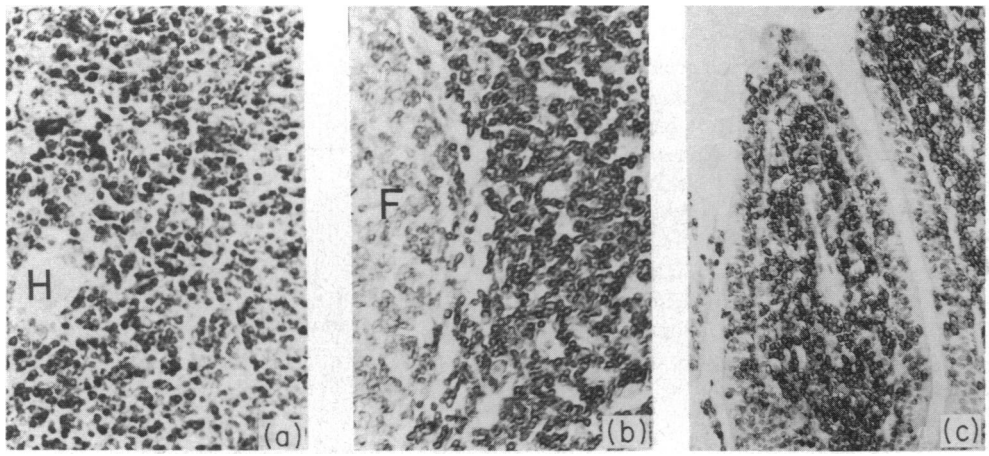

Figure 2. Thymus (a), lymph node (b) and small intestine (c) tissue sections stained by 1.11.32. All lymphocytes in thymus and lymph node are positive for LCA. There is a heavy infiltration of the epithelium of the small intestine with lymphocytes. F = follicle; H = Hassall's corpuscle. Magnification: (a), (b) × 320; (c) × 250.

Day 60–70. The infiltration of the intestinal wall with antigen-positive cells occurred after Day 120; however, the density of positive cells was much lower than that found in postnatal intestine.

Immunochemical characterization

The molecules precipitated from thymus lysates had apparent molecular weights of 190,000 (main band) and 210,000 when run under reducing conditions, while those from lymph node lysates had apparent molecular weights of 225,000, 210,000 and 190,000 (Fig. 4). Labelling of sialic acid residues using tritiated sodium borohydride, followed by immunoprecipitations and fluorography, revealed bands of similar molecular weights to the above (data not shown) and demonstrated that these antigens were glycoproteins. Western blots of membrane proteins from spleen, thymus and lymph node (our unpublished data) revealed that each of the protein bands immunoprecipitated are stained by the antibodies, and that the antigenic site recognized is common to all forms.

DISCUSSION

The monoclonal antibodies (1.11.32 and 1.28.124) described in this study define an antigen which, based on tissue distribution, is an analogue of the LCA of the rat (Fabre & Williams, 1977; Sunderland, McMaster & Williams, 1979), man (Dalchau *et al.*, 1980; Ishii *et al.*, 1983), and also of the Ly 5 antigen of the mouse (Scheid & Triglia, 1979; Scheid *et al.*, 1982). In the sheep, this antigen is present only on cells of haemopoietic lineage, being present on all lymphocytes of the thymus, spleen, lymph node and peripheral blood, as well as on macrophages and granulocytes. The antigen is absent from mature fetal and adult erythrocytes, but its status with respect to immature cells of the erythrocyte lineage is unknown. The murine Ly 5 antigen is present on nucleated erythrocyte precursors, while being absent from mature fetal and adult erythrocytes (Scheid *et al.*, 1982).

Cells both from and within different haemopoietic lineages express different molecular forms of this antigen in the rat, mouse and man (Brown & Williams, 1982; Spickett *et al.*, 1983; Michaelson, Scheid & Boyse, 1979; Coffman & Weissman, 1981; Dalchau & Fabre, 1981). B lymphocytes and the suppressor subset of T lymphocytes bear the high MW form of 220,000 in the mouse and rat (Scheid *et al.*, 1982; Spickett *et al.*, 1983). In addition to a common form found on all T lymphocytes of 200,000, molecular forms of 210,000 are also found on Lyt 2+ cells in the mouse (Tung, Scheid & Palladino, 1983). Different molecular forms are also present at different stages of maturation and differentiation (Sarmiento *et al.*, 1982). Conservation of structure between species is also apparent as monoclonal antibodies to the mouse B lymphocyte form cross-react with human cells

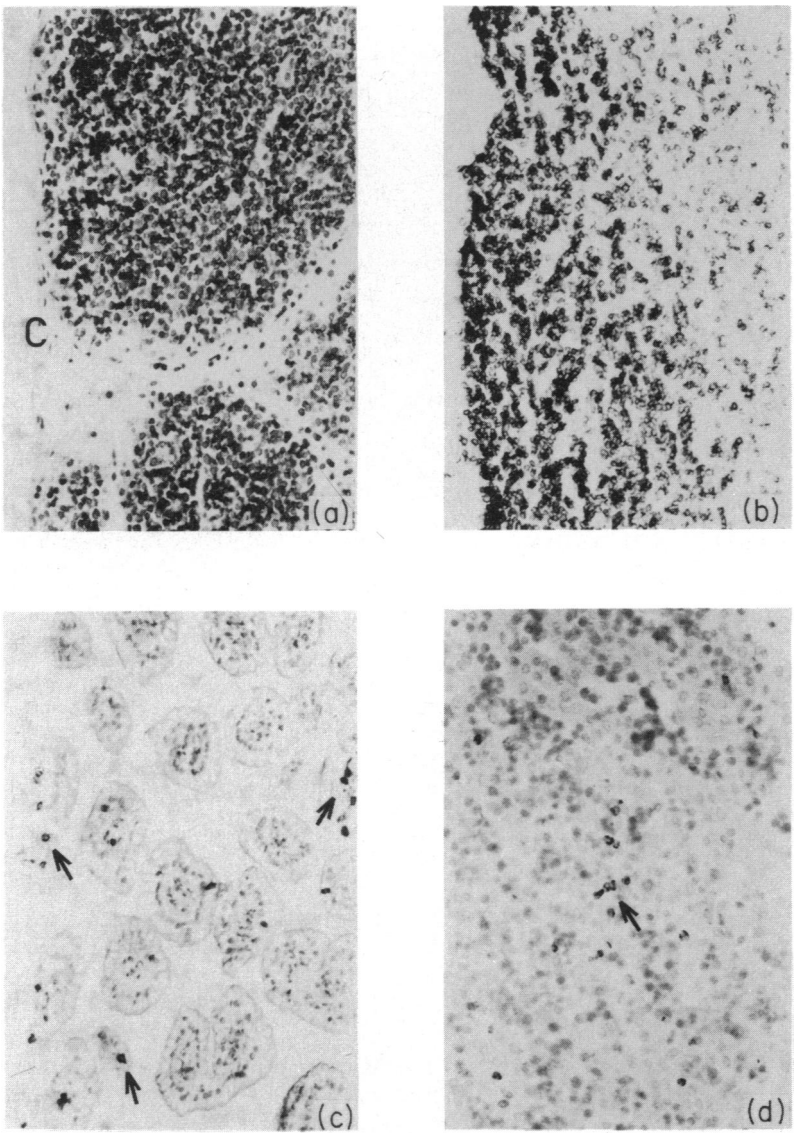

Figure 3. Fetal tissue sections stained by 1.11.32 (a) thymus Day 40): all lymphocytes are stained but there is no differentiation into cortex and medulla (C = capsule); (b) mesenteric lymph node (Day 120): relatively undifferentiated, no follicles, outer cells more heavily stained; (c) small intestine (Day 120); a small number of positive cells are present in the epithelium, indicated by arrows; (d) liver (Day 27–30): a small number of positive cells of haemopoietic morphology are present (the arrow points to a foci of positive cells). Magnification: (a), (c), (d) × 250; (b) × 200.

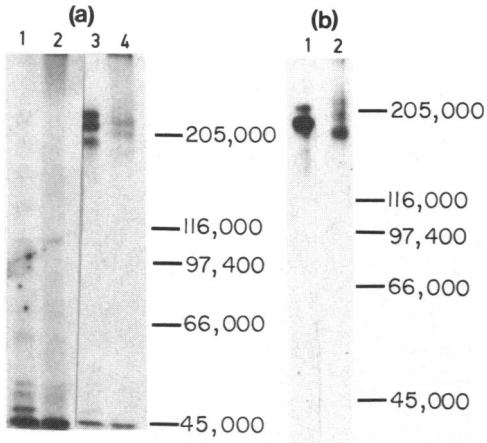

Figure 4. 6% SDS-PAGE of LCA immunoprecipitated from ^{125}I-labelled cells from (a) lymph and (b) thymus. (a) The monoclonal antibodies used were as follows. Tracks 1 and 2; 17.3 (a monoclonal antibody recognizing another sheep lymphocyte antigen); Tracks 3 and 4; 1.11.32. Tracks 1 and 3 were reduced, Tracks 2 and 4 were not reduced. (b) The monoclonal antibody used was 1.11.32. Track 1 was reduced, Track 2 was not reduced. MW standards from top to bottom: myosin 205,000; galactosidase 116,000; phosphorylase *b* 97,400; BSA 66,000; ovalbumin 45,000.

(Scheid *et al.*, 1982). The monoclonal antibodies described here recognized a common form of this antigen in the sheep, as shown by its presence on all lymphocytes, macrophages and granulocytes, as well as the range of MW forms detected. No cross-reaction was found with mouse, rat, man or chicken, but the antigen is present on bovine cells.

The function of this antigen is currently unknown. Antibodies to Ly 5 have been shown to suppress both T- and B-lymphocyte function *in vitro* (Yakura *et al.*, 1983; Harp & Ewald, 1983). Studies on the ontogeny of Ly 5 and the T200 equivalent antigen have been performed in the mouse (Triglia, 1980; Van Ewijk, Jenkinson & Owen, 1982; Scheid *et al.*, 1982). In fetal mice Ly 5 is detected as early as Day 8 of gestation in the yolk sac and Day 12 in liver, these being the earliest ages studied (Triglia, 1980). T200 is present on Day 14 in the thymus, while Lyt-1 and Lyt-2 do not appear until Day 14 in the thymus, while Lyt-1 and Lyt-2 do not appear until Day 15 and Day 17, respectively (Van Ewijk *et al.*, 1982). The B-cell form of LCA is one of the earliest B markers expressed (Scheid *et al.*, 1982).

The long gestation period of the sheep enables a more detailed study of the appearance of the various differentiation antigens. Preliminary studies with 1.11.32 and 1.28.124 have shown that a population of cells in Day 27–30 sheep fetal liver express this antigen (earliest age examined), while most lymphocytes from Day 40 onwards in thymus express this antigen. Further studies on the ontogeny of this antigen in the sheep fetus are being undertaken.

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