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Laboratory techniques

KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections

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SUMMARY A new monoclonal antibody, KP1, raised against a lysosomal fraction of human lung macrophages, recognises a fixation-resistant epitope in a wide variety of tissue macrophages (such as Kupffer cells germinal centre, splenic, and lamina propria macrophages), and in granulocyte precursors. Its broad reactivity with cells of the mononuclear phagocytic lineage was established by testing on routinely processed samples of normal and reactive lymphoid tissues. Interdigitating reticulum cells were unstained or showed limited cytoplasmic staining while Langerhans' cells and follicular dendritic reticulum cells were unreactive. KP1 recognises a molecule of about 110 kilodaltons in macrophage-rich human tissue when tested by either immunoprecipitation or Western blotting (although the latter procedure also shows two additional components with molecular weights of 70 and 40 kilodaltons).

KP1 should be of considerable value for studying disorders of the monocyte/macrophage system, including both reactive and neoplastic states (such as true histiocytic proliferations).

Monocytes, macrophages, and other elements of the mononuclear phagocytic system have several vital roles in healthy subjects, including antigen processing, secretion of a wide range of molecules, and phagocytosis.[1] Despite their obvious importance progress in defining antigenic molecules specific for these cells which can be used to trace their distribution in normal and pathological human tissue has been limited. This emerged from the analysis of anti-myeloid antibodies performed in the Third International Workshop on Leucocyte Differentiation Antigens[2] which defined several new molecules on mononuclear phagocytes according to the CD system (CD31, CD32, CD35, CD36). Many of these markers, however, are also expressed on other tissues such as CD26 on platelets and CD31 on endothelium, or are absent from, or present at only low levels on, tissue macrophages. None of these new antigens, nor any of the CD antigens reactive with monocytes identified at earlier workshops (CD11, CD13, CD14, CD16 or CD18) could be categorised as "pan-macrophage" and the nearest approach to the definition of such markers in the Third Workshop lay in the identification, by staining tissue sections, of a group of five antibodies which showed closely similar reactions, and which all stained selectively most tissue macrophages (antibodies 24, Y2/131, Ki-M6, Y1/82A, EBM11).

The molecular targets for these antibodies remain poorly defined, however, and all of these reagents suffer from the practical limitation, in the context of diagnostic pathology, of not reacting with routinely processed paraffin wax embedded tissue. In this paper we describe a new monoclonal antibody (KP1) which recognises most human macrophages in routinely processed tissue, and for which the target molecule can be identified by immunocytochemical techniques.

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Material and methods

PREPARATION OF ANTIGEN

Samples of fresh normal human lung, removed during surgery for lung carcinoma, were obtained from this hospital. Cells were extracted by irrigating the lung tissue with HEPES buffered RPMI 1640 medium containing 10% fetal calf serum (FCS, Gibco Biocult, Long Island Co, Ltd). The resulting washings were spun down and separated on a Lymphoprep

(Nygaard) density gradient. Macrophages accounted for about 80% of the total white cell count. These cells were washed and a total of 4×10^8 resuspended in 1·5 ml 0·34M sucrose containing 1 mM iodoacetamide, 0·2 mM phenylmethyl sulphonyl fluoride, 0·5% sodium azide, 10 mM leupeptin and Pepstatin A (Sigma Chemical Co.). The suspension was sonicated and the nuclei pelleted by centrifugation at 300 *g* for five minutes. The supernatant was then spun at 8000 *g* for 15 minutes and the resulting supernatant stored at −70°C. This material constituted the antigen used for immunisation.

IMMUNISATION SCHEDULE AND FUSION

Balb/c mice were immunised three times intraperitoneally at eight day intervals with 50 μg antigen emulsified in Freund's complete adjuvant. A fusion was carried out three days after the last injection according to a previously described technique[3] and supernatants were screened initially on cryostat sections of human lung and tonsil. Further testing was carried out on paraffin wax sections of lung tissue fixed in formol saline.

CELLS AND CELL LINES

Routine bone marrow smears taken for diagnostic purposes and smears of human peripheral blood were obtained from the haematology department. Slides were air-dried overnight and stored at −20°C. They were fixed before use in a variety of different fixatives, including buffered formol acetone and acetone methanol, as detailed elsewhere.[4]

TISSUE SECTIONS

Normal tonsil was obtained from the Ear, Nose and Throat Department of the Radcliffe Infirmary, Oxford.

Fixed tissue: Tissues obtained fresh were fixed in unbuffered formol saline before paraffin wax embedding. Routinely processed paraffin wax embedded tissue biopsy specimens were obtained from the histopathology department. All tissues had been fixed in formol saline, with the exception of bone marrow trephine biopsy specimens which were fixed in formol saline containing 1% glacial acetic acid. Routine sections from several other hospitals which had been fixed in B5 fixative were also analysed.

Fresh tissues: Tonsil samples were snap frozen in liquid nitrogen. Cryostat sections of 6 μm thickness were cut, dried, fixed and stored as previously described.[5]

IMMUNOENZYMATIC LABELLING

Tissue sections and cell smear preparations were stained using the alkaline phosphatase:anti-alkaline phosphatase (APAAP) method.[5,6] Before immunostaining, paraffin wax sections of formalin fixed tissue were dewaxed, hydrated, and then incubated for 20–30 minutes in 0·1% trypsin solution in 0·1% calcium chloride solution (pH 7·8).

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOTTING

Fresh spleen tissue in which there was a heavy macrophage infiltration was obtained after splenectomy from a case of B cell lymphoma. The tissue was stored frozen at −70°C until use. Samples were homogenised in a hypotonic 20 mM Tris (pH 7·5) buffer, the resulting suspension centrifuged at 70 000 *g* for 20 minutes, and the supernatant removed. The pellet was rehomogenised in the Tris buffer, this time containing 2% Tween 40 (Sigma Chemical Co.) and spun at 4000 *g* to remove nuclei before a high speed 70 000 *g* spin. The 70 000 *g* supernatant was retained and the pellet was washed with 20 mM Tris buffer. The washed pellet was rehomogenised in the Tris buffer containing 1% Nonidet P40 (NP40), spun at 70 000 *g*, and the supernatant retained. A pellet of 2×10^8 U937 cells was lysed in 1% NP40. After a 70 000 *g* spin the supernatant was collected.

All procedures were performed at 4°C in the presence of the metabolic inhibitors described above.

The three different spleen lysates, the U937 lysate, and the antigen used for immunisation were run on a 7·5% SDS polyacrylamide gel slab.[7] Proteins were transferred electrophoretically overnight by Western blotting[8] on to nitrocellulose paper using a TRANS-BLOT apparatus (Biorad). After blocking free protein binding sites with bovine serum albumin (BSA) in TBS (0·5M Tris-HCl, pH 7·6, diluted in 1:10 in 1·5M saline) the nitrocellulose paper was incubated for 30 minutes in KP1 antibody or in an irrelevant monoclonal antibody. After washing, the APAAP staining procedure was then completed.[6] Molecular weight standards transferred on to nitrocellulose paper were stained with 0·1% Fast Green[9] to calibrate the blots.

IMMUNOPRECIPITATION

A crude membrane preparation from a spleen rich in macrophages was solubilised in 2% NP40 and the preparation enriched for glycoproteins by lentil lectin affinity chromatography. The 1M α-methyl mannoside eluate from the lectin column was dialysed and a sample (100 μg) was labelled with 1mCi[^{125}I] iodide (Amersham) in the presence of 20 μg Iodogen (Pierce).

Labelled material was gel filtered to remove unbound iodine and precleared twice with affinity purified rabbit anti-mouse Ig antibody bound to *Staphylococcus aureus*. Monoclonal antibodies (in the form of undiluted hybridoma culture supernatants) were mixed with the pre-cleared extract, and after one

hour rabbit anti-mouse Ig bound to *S aureus* was added. After a further hour the immunoabsorbent was washed three times in buffer containing 1% NP40 and 500 mM sodium chloride. Immunoprecipitates were analysed by SDS-PAGE followed by autoradiography of the dried gel using an enhancing screen.

FACS ANALYSIS

White cells were obtained either by centrifugation on Trisil-Ficoll or by red cell lysis of whole blood. In the latter procedure samples of normal peripheral blood were treated for 12 minutes with red cell lysing solution consisting of ammonium chloride (8·29 g/l), sodium edetic acid (0·37 mg/ml), and potassium bicarbonate (1·0 g/l). The resulting white cells were washed four times in Dulbecco "A" medium (Oxoid code BR 14a) containing 2·5% normal human serum and then incubated for 30 minutes at 4°C with KP1 antibody (undiluted hybridoma culture supernatant). After washing twice, the cells were incubated for a further 30 minutes with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Sigma code F-2012) diluted 1/20. The cells were washed twice in 1·5% formaldehyde in PBS and analysed in a FAC-Scan (Becton Dickinson) flow cytometer using an argon ion laser of 15mW at 488 nm. Lymphocyte, granulocyte, and monocyte populations were analysed separately by gating on forward and side scatter measurements.

Results

On preliminary screening of the hybridomas obtained using spleen cells from a mouse immunised with the human macrophage antigen, 18 supernatants stained macrophages selectively in cryostat sections of human lung and tonsil. Only one supernatant, however, stained most macrophages in these tissues. This supernatant also stained macrophages in paraffin wax sections of lung tissue fixed in formol saline. The cells producing this supernatant were cloned to produce the cell line KP1.

BIOCHEMICAL CHARACTERISATION OF THE KP1 ANTIGEN

Immunoprecipitation of a radiolabelled homogenate of macrophage-rich human spleen showed a protein band with a molecular weight of about 110 kilodaltons (fig 1).

The results of Western blotting are shown in fig 2. Under reducing conditions KP1 stained three bands with molecular weights of 110, 70, and 40 kilodaltons in the lung antigen preparation and in NP40 lysates of U937 cells and spleen. The bands consistently detected in several different experiments were relatively diffuse. No staining of material at these positions was seen in control blots stained with an unrelated monoclonal antibody. Under non-reducing conditions KP1 reacted with an additional band of 220 kilodaltons in the NP40 spleen lysate (fig 2), and in these preparations the 70 and 40 kilodalton bands were less intense. The two spleen lysates prepared in the absence of detergent or in the presence of Tween 40 gave identical results on Western blotting with those obtained using the NP40 lysate.

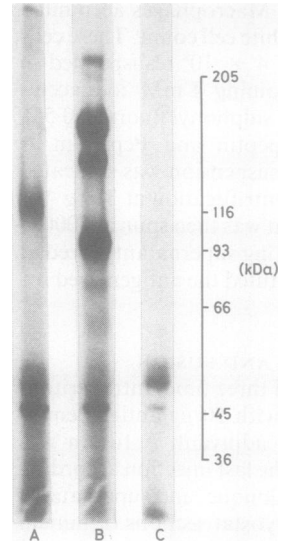

Fig 1

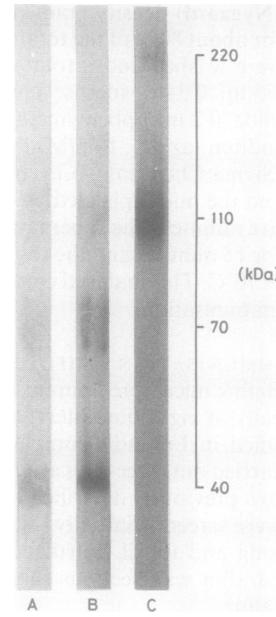

Fig 2

Fig 1 *Immunoprecipitation of a ^{125}I labelled lysate of human macrophage-rich tissue. Track A: prominent band about 110 kilodaltons is precipitated by antibody KP1. Track B: antibody MHM23 precipitates LFA-1/Mac-1 (CD11/18) complex but no material with the same molecular weight as the KP1 antigen. Track C: control immunoprecipitation in which no antibody was used.*

Fig 2 *Western blotting of the KP1 antigen. Lanes A and B show 110, 70, and 40 kilodalton bands detected by KP1 under reducing conditions in extracts of lung (lane A) and U937 cells (lane B). An additional band (220 kilodaltons) is detected in the spleen lysate under non-reducing conditions (lane C), and also a decrease of the 70 kilodalton band and absence of the 40 kilodalton band.*

REACTIONS OF KP1 ON TISSUE SECTIONS

The results of immunocytochemical studies with KP1 antibody on routinely processed sections of normal tissues are summarised in the table. Trypsinisation of tissue sections fixed in formol saline before staining considerably enhanced the intensity of the labelling

Table *Reactivity of antibody KP1 with routinely processed paraffin wax embedded tissues*

Tissues	*Cell types stained**
Lymphoid tissues:	
Bone marrow	Macrophages and myeloid precursors
Tonsil and lymph node	Germinal centre macrophages, interfollicular macrophages, sinus macrophages and small mononuclear cells in T cell areas
Spleen	Germinal centre macrophages and red pulp macrophages
Thymus	Medullary and cortical macrophages
Non-lymphoid tissues:	
Lung	Alveolar macrophages
Kidney	Interstitial macrophages
Liver	Kupffer cells
Skin	Dermal macrophages
Pancreas	Interstitial macrophages
Gut	Lamina propria macrophages
Uterus	Tissue macrophages
Testis	Tissue macrophages
Thyroid	Tissue macrophages
Bone	Osteoclasts

*Staining in all cells was diffusely present in the cell cytoplasm.

obtained with KP1. Trypsination was not required when staining tissues fixed in B5 fixative.

Normal tissue

The antibody reacted with tissue macrophages in a wide range of tissues, including lung macrophages (fig 3), germinal centre macrophages (fig 4), Kupffer cells in the liver (fig 5) and bone marrow macrophages (fig 6). In the latter tissue the antibody also reacted with myeloid precursors (fig 6), and this reaction was confirmed by staining smears of human bone marrow in which myelocytes and also many mature granulocytes were strongly labelled. Megakaryocytes and cells of erythroid lineage were not labelled by KP1 in smears or sections of bone marrow.

In the spleen KP1 antibody labelled macrophages in germinal centres and in red pulp. No staining of marginal zone macrophages was seen. In the thymus KP1 stained both medullary and cortical macrophages. Langerhans' cells, interdigitating reticulum cells, and follicular dendritic cells were all unstained, with the exception (as detailed below) of a few of the interdigitating reticulum cells seen in dermatopathic lymphadenopathy. Tissue polymorphs were usually not stained by KP1 or showed only weak labelling. No staining was seen of any non-haemopoietic cell types (such as epithelium, muscle, etc.).

Reactive lymphoid tissues

A variety of reactive lymph nodes (comprising at least two examples of follicular hyperplasia, sinus histiocytosis, sarcoidosis, tuberculosis, cat scratch disease, dermatopathic lymphadenopathy and toxoplasmosis) were studied and broad reactivity against cells of macrophage lineage was observed, epithelioid granulomas and multinucleated cells being particularly strongly labelled (figs 7 and 8). Most of the abnormal interdigitating reticulum cells characteristic of dermatopathic lymphadenopathy were negative (fig 9), although a minority of these cells showed weak positivity, usually localised to a small region close to the nucleus.

REACTIONS OF KP1 ON BLOOD CELLS

KP1 stained all monocytes and most granulocytes in smears of peripheral blood. The staining of monocytes was granular in appearance and suggested an intracytoplasmic location of the antigen, possibly associated with lysosomal granules. Analysis by flow cytometry showed strong reactivity of all monocytes with KP1 when white cells were prepared by the whole blood lysis method, but not when white cells separated on lymphoprep were analysed. Granulocytes gave a weak or negative reaction while lymphocytes and platelets were unstained.

Discussion

As the antigen recognised by KP1 is resistant to conventional fixatives such as formalin and B5, this antibody may be of practical importance for diagnostic pathologists. KP1 seems to recognise an antigen which persists throughout cell maturation from the monocyte to the mature macrophage stage. Indeed, the amount of KP1 antigen present in macrophages may increase when these cells are activated and mature, as indicated by the greater intensity of staining of tissue macrophages compared with that seen in peripheral blood monocytes. This finding is in contrast to that obtained for myeloid cells, in which the KP1 antigen is weaker in mature granulocytes than in the bone marrow precursors of these cells.

The only KP1 positive cells in lymphoid tissue sections other than classic macrophages were the intermediate sized rounded cells seen in T cell areas. These were shown in subsequent studies to correspond to the cell type known as "plasmacytoid T cells", and their reaction with antibody KP1 provides additional evidence that they are related to monocytes/macrophages.[10]

If the reactions of antibody KP1 are compared with those of previously published monoclonal anti-macrophage antibodies, the greatest similarity is with antibodies Y1/82A[11], EBM11[12], Ki-M6[13] and Ki-M7[14]. The first three of these antibodies were clustered into a possibly homogeneous group in the Third International Workshop on Leucocyte Differentiation Antigens[2] on the basis of their "pan-macrophage" pattern of reactivity on tissue sections. There are, however, minor differences between the KP1 and the reactions of those other monoclonal antibodies, which suggest that they may recognise different antigens.

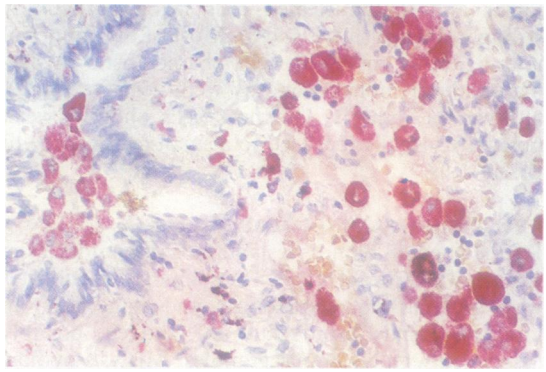

Fig 3 *Labelling of alveolar macrophages by antibody KP1. Immunostaining in this figure and in figs 4–9 was performed by the APAAP technique on trypsinised paraffin wax sections.*

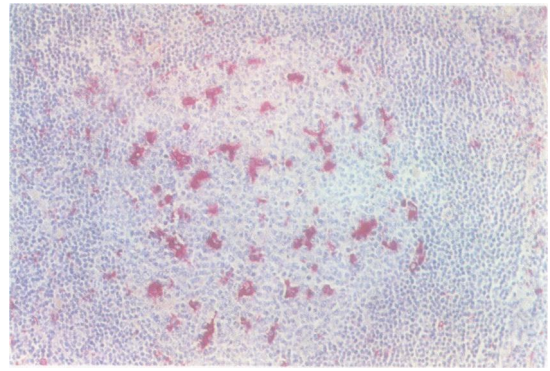

Fig 4 *Labelling of tingible body macrophages in reactive lymphoid tissue with antibody KP1.*

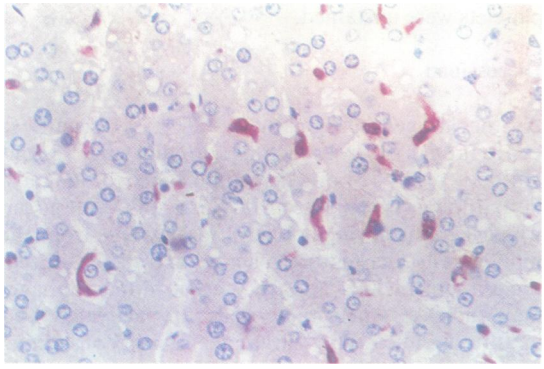

Fig 5 *Staining of Kupffer cells in the liver by antibody KP1.*

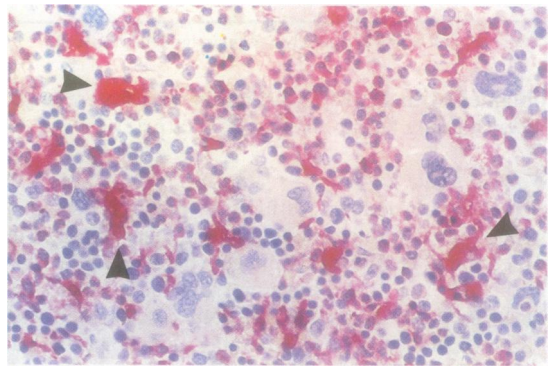

Fig 6 *Strong labelling of macrophages (arrowed) in a bone marrow trephine biopsy specimen by antibody KP1 and weaker staining of many myeloid precursors.*

Antibodies Ki-M6 and Ki-M7, for example, were reported as reacting with antigens which differ in molecular weight (60 and 29 kilodaltons, respectively) from the molecules detected by KP1.[13,14] EBM11 antibody shows some similarity to KP1 in that it has been reported as reacting by Western blotting with molecules of 120, 70, 64 and 22 kilodaltons.[12] EBM11 antibody is reported to react in tissue sections with interdigitating reticulum cells and Langerhans' cells, however, whereas KP1 does not seem to react with these cell types.[12,15]

On the basis of published data it seems that KP1 is different in terms of the molecule(s) which it recognises and in its immunocytochemical reactions, from previously reported anti-macrophage monoclonal antibodies.[16,17] It should be noted, however, that the molecular weights of the target antigens reported for the monoclonal antibodies which it resembles most closely in its staining reactions—that is, Y1/82A, Ki-M6, Ki-M7 and EBM11—have not been independently validated by other laboratories; and indeed, a result has been reported in immunoprecipitation experiments for Ki-M6 (90 kilodaltons) which differs from the values obtained by Western blotting.[2] Furthermore, we have not attempted a direct comparison between the immunocytochemical reactions of these other antibodies and those of antibody KP1, and the published reactions of these antibodies were obtained primarily using cryostat sections (in which antigens are usually better preserved than in paraffin wax sections), whereas KP1 was evaluated on formalin fixed tissue. Further analysis of the antigens recognised by all of these antibodies is therefore required if their association is to be established with certainty.

Interestingly, the material detected by KP1 antibody by immunoprecipitation shows a diffuse pattern on gel electrophoresis, suggesting that it may be extensively glycosylated. The finding that antigenic material of the same molecular weight was detected by KP1 in both detergent-free spleen lysates and in those

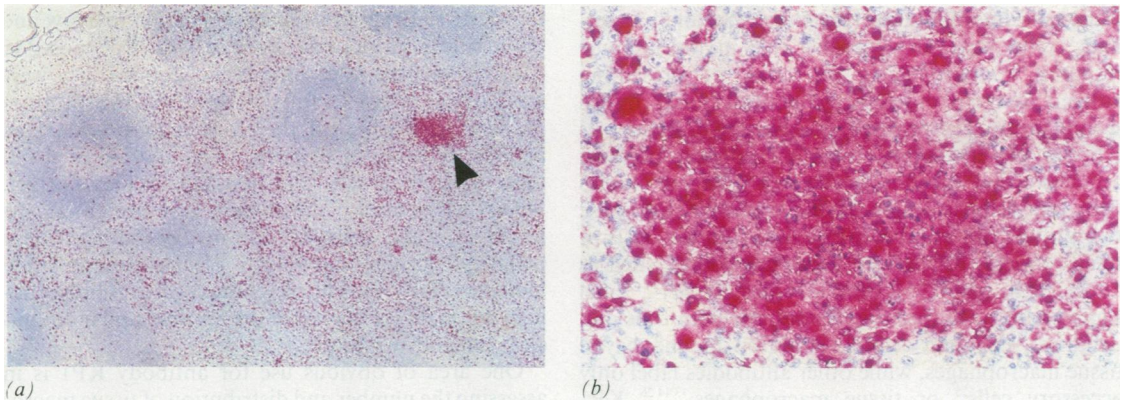

Fig 7 *Cat scratch disease biopsy specimen stained with antibody KP1. A cluster of macrophages is arrowed in (a) and shown at higher power in (b).*

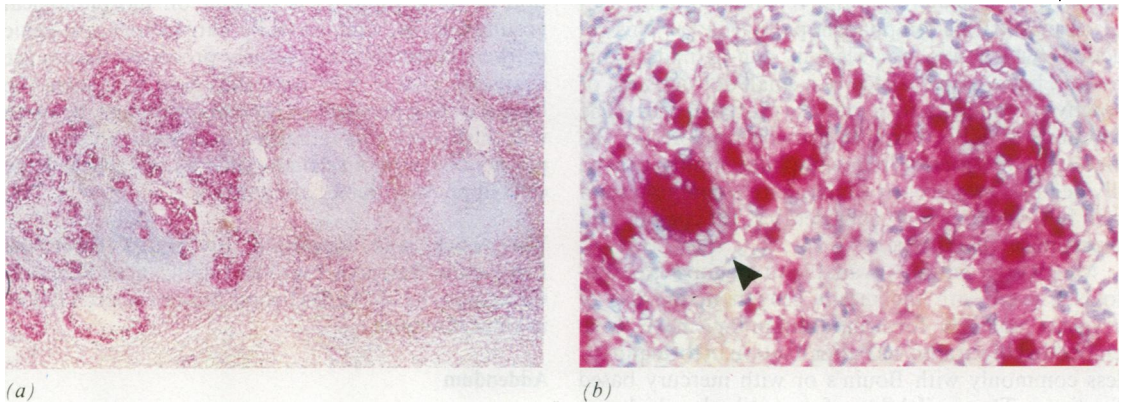

Fig 8 *Sarcoidosis in human spleen. (a) KP1 positive granulomata around a lymphoid area; (b) granuloma at higher magnification, with a multinucleate giant cell (arrowed).*

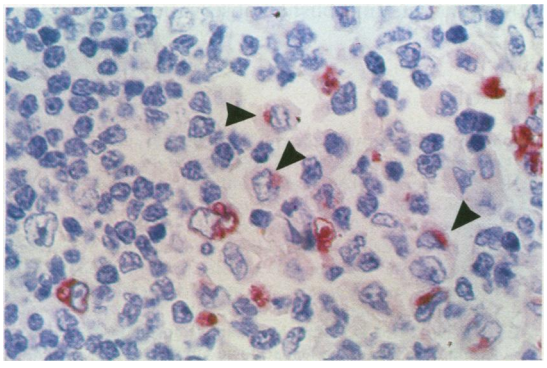

Fig 9 *Dermatopathic lymphadenopathy showing dot-like reactivity with antibody KP1 in some atypical interdigitating reticulum cells (arrowed).*

containing Tween 40 or NP40 indicates that the antigen is probably present in the cytosol, microsomal, and surface membrane fractions of macrophages. This raises the possibility that, as found for other monocyte/macrophage antigens,[18–20] the KP1 antigen is present in lysosomes, the contents of which move to the cell surface and to the extracellular environment during exocytosis. The fact that the separation procedure used for isolating human blood white cells influenced whether KP1 antigen could be detected on the cell surface by flow cytometry also supports this view.[19] Further work is needed to elucidate the biochemical nature of the KP1 antigen and to define in detail its subcellular localisation.

Functional studies have indicated that the monocyte/macrophage family can be divided into two groups. The first group, comprising follicular den-

dritic cells, interdigitating reticulum cells, and Langerhans' cells, have an accessory role in the immune response,[21 22] particularly in terms of presenting antigen to lymphoid cells. In contrast, cells in the second group, comprising tissue macrophages such as Kupffer cells and lung alveolar macrophages, perform a microbicidal and phagocytic role.[23] Recent studies with monoclonal antibodies have shown that these two groups can also be distinguished on the basis of their antigenic differences. Some antibodies, such as anti-CD11c (p150,95) EBM11[15] and Ki-M1[24], label Langerhans' cells, interdigitating reticulum cells and dendritic reticulum cells, in addition to phagocytic tissue macrophages, while other antibodies label only accessory cells[25] or tissue macrophages.[13 14 26] KP1 therefore seems to be another anti-macrophage antibody which reacts with phagocytic tissue macrophages but not with accessory cells. The only exception lies in its reaction with interdigitating reticulum cells in cases of dermatopathic lymphadenopathy (fig 9). This reaction (which was not seen in non-dermatopathic nodes), however, consisted of a small dot-like area of reactivity confined to a minority of the cells. The reactions of KP1 therefore provide support for the view that the monocyte/macrophage family can be divided into two groups which share a common stem cell but which show considerable functional and biochemical divergence.[27 28]

Most tissue samples available to diagnostic pathologists, even with the recent growth in the use of cryostat sections for immunostaining, have been embedded in paraffin wax after fixation in formalin (or less commonly with Bouin's or with mercury based fixatives). The availability of an antibody which can detect macrophages in this type of material is therefore of considerable potential in the context of routine histopathology. We are aware of only two other monoclonal antibodies recognising macrophage associated antigens in conventionally processed paraffin wax embedded tissue, and each of these is less suitable for routine use than KP1 antibody. Ki-M7 antibody is described as staining paraffin wax sections but this reactivity is said to be inconsistent, macrophages sometimes being weakly stained or unlabelled.[14] The other antibody, MAC387, is restricted in its reactions to a subpopulation of tissue macrophages, being unreactive with germinal centre macrophages or Kupffer cells, and it also labels squamous epithelium and granulocytes strongly.[29 30]

It may be added that the analysis of the pattern of immunocytochemical reactivity of KP1 in this study has been performed almost entirely on routinely processed tissue. It is likely that in this tissue there is some reduction in antigenic reactivity, and cells with low levels of antigen expression may therefore appear artefactually negative. This is suggested by the fact that KP1 stains granulocytes strongly in marrow and blood smears (which have been only briefly exposed to fixative) whereas in routine tissue sections they were weak or unreactive. A full description of the spectrum of KP1 reactivity will therefore require systematic analysis of cryostat tissue sections and cultured cell lines of haemopoietic and non-haemopoietic origin. Nevertheless, even if the antigen were present at low levels in some cells of non-myeloid/mononuclear phagocyte origin, the results of the present study indicate clearly that in the context of routine pathology it is highly selective for the myeloid and mononuclear phagocyte lineage.

One area of obvious use for antibody KP1 is in assessing the number and distribution of tissue macrophages in pathological conditions (such as inflammation and neoplasia). It may also be of value in the diagnosis of malignant histiocytic tumours. The detection of these tumours is difficult on morphological grounds alone, and no fully satisfactory antigenic markers detectable in routinely processed tissues are available. Use of KP1 may thus help to resolve the long standing problem of how to distinguish between true histiocytic malignancies and high grade neoplasms of lymphoid origin (such as anaplastic large cell lymphoma/"Ki-1 lymphoma").

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Addendum

Experiments performed in the authors' laboratory since the submission of this paper have shown that the monoclonal anti-macrophage antibodies Y1/82A,[11] EBM11,[12] and Ki-M6[13] all recognise the 110 000 kilodalton glycoprotein detected by antibody KP1 (Micklem *et al*, unpublished observations).

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