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A 72-kD B cell-associated surface glycoprotein expressed at high levels in hairy cell leukaemia and plasma cell neoplasms

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SUMMARY

The present paper describes two new MoAbs, GHI/75 and VMP55, which were raised against a glycoprotein enriched lysate of hairy cell leukaemia. These antibodies recognized a new antigen of 72 kD (unreduced) and 83 kD (reduced) molecular weight. GHI/75 and VMP55 gave very strong staining of plasma cells, moderate labelling of circulating B cells but only weak staining of monocytes, some tissue macrophages and lymphoid cells. Neither antibody reacted with neutrophils or any non-haematopoietic cells. Both antibodies, however, strongly labelled the tumour cells in hairy cell leukaemia, multiple myeloma, plasmacytoma and lymphoplasmacytic lymphomas. No staining was seen of the neoplastic cells in Hodgkin's disease, myeloid leukaemia or T cell lymphomas. The two antibodies, GHI/75 and VMP55, may be of value in the differential diagnosis of hairy cell leukaemias and plasma cell neoplasms. In addition, the ease with which their antigen can be purified provides the possibility for a detailed study of this molecule.

Keywords hairy cell leukaemia monoclonal antibodies antigen immunohistochemistry

INTRODUCTION

Hairy cell leukaemia is characterized by a chronic course, splenomegaly and the presence of neoplastic cells of characteristic morphology in the bone marrow, peripheral blood and spleen. Although in the past there were suggestions that hairy cell leukaemia cells might be of monocyte (Scheinberg *et al.*, 1976) or T cell origin (Saxon, Stevens & Golde, 1978), it is now accepted on the basis of its pattern of antigen expression, e.g. CD19, CD20 and CD22 (Cohen, George & Kremar, 1979; Hsu, Yang & Jaffe, 1983; Falini *et al.*, 1985) and immunoglobulin gene rearrangement studies (Cleary *et al.*, 1984), that hairy cell leukaemia is of B cell origin.

The correct diagnosis of hairy cell leukaemia is important since it can be treated with α-interferon (Samuels, Golomb & Brownstein, 1987) or deoxycoformycin (Kraut, Bouroncle & Grever, 1986). In most cases the neoplastic cells can be identified by their morphology (Schrek & Donnelly, 1966) and the presence of tartrate-resistant acid phosphatase (TRAP) (Yam, Li & Lam 1971). However, some cases of hairy cell leukaemia are TRAP-negative, and neoplastic cells in some other types of lymphoproliferative disorder can resemble hairy cell leukaemia both clinically, cytologically and may even contain the enzyme TRAP (Katayama & Yang, 1977; Palutke *et al.*, 1981). MoAbs have therefore proved to be of great value for the differential diagnosis of hairy cell leukaemia (Posnett, Chiorazzi & Kunkel, 1982; Hsu *et al.*, 1983; Posnett *et al.*, 1984; Falini *et al.*, 1985; Schwarting, Stein & Wang, 1985; Visser *et al.*, 1989.). Not only do they serve to confirm the diagnosis, but they may also be of value during treatment for monitoring the presence of residual disease (Kristensen, Ellegaard & Hokland, 1987; Vardiman *et al.*, 1988).

The present paper describes two MoAbs which recognize a molecule present at high levels on hairy cell leukaemia cells. These antibodies represent an addition to the panel of reagents which can be used in the diagnosis of hairy cell leukaemia. Furthermore, since they could be used by affinity chromatography to purify microgram amounts of the target antigen from hairy cell leukaemia cells, it should easily be possible to isolate enough of this new B cell-associated molecule to characterize it in detail.

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MATERIALS AND METHODS

Antigen and immunization

Fresh spleen was obtained after a splenectomy from a case of hairy cell leukaemia. The tissue was homogenized in 2·5% Tween 40 in 20 mM Tris pH 7·6 containing 1 mM iodoacetamide, 100 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM phenyl methyl sulphonyl flouride (Sigma Chemical Co., St Louis, MO). The homogenate was spun at 300 *g* for 10 min to remove nuclei and large debris and then spun at 70 000 *g* to sediment the membrane fraction. The pellet was then solubilized in 2% Non-idet P40

(NP40) in extraction buffer and applied to a lentil lectin-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ). After washing with 20 mM Tris containing 250 mM NaCl, the material bound to the column was eluted using 1 M methyl mannoside in extraction buffer and dialysed against the extraction buffer. All solutions used contained 1% NP40. Antigen was precipitated using trichloroacetic acid, washed and dialysed against phosphate buffered saline (PBS). BALB/c mice were injected three times intraperitoneally at 8-day intervals with the antigen emulsified in Freund's adjuvant.

MoAb production

Two fusions were carried out 3 days after the last immunizations. The spleens were disaggregated and incubated with NS1 (P3-NS1/1 Ag 4.1) myeloma cells in 50% (v/v) polyethylene glycol 4000 (PEG 4000, Prolabo, France) for 90 min at 37°C as described by Orlik & Altaner (1988). After washing, the remainder of the fusions were performed, as was subsequent cloning and production of antibody as ascitic fluid, using a previously described technique (Mason, Cordell & Pulford, 1983). Initial screening of the supernatants was carried out on cryostat sections of a hairy cell leukaemia spleen. Further testing was carried out using cryostat sections of human tonsil and cytocentrifuge preparations of peripheral blood.

Preparation of cells and tissue sections

Normal peripheral blood cells were separated on Lymphoprep (Nyegaard, Oslo, Norway) and cytocentrifuge preparations were made. Heparinized leukaemic peripheral blood samples were obtained from patients attending the John Radcliffe Hospital, Oxford, and the Royal North Shore Hospital, Sydney, Australia. The samples were used to make either blood smears or cytocentrifuge preparations of mononuclear cells. The slides were stored and fixed as previously described (Erber, Pinching & Mason, 1984).

Normal tonsil was obtained from the E.N.T. Department of the Radcliffe Infirmary, Oxford. Some tonsil samples were minced to produce cell suspensions from which mononuclear cells were obtained using Lymphoprep. Other normal tissue and lymphoma samples were obtained from the Histopathology Department of the John Radcliffe Hospital and snap frozen in liquid nitrogen. Cryostat sections of 6 μm thickness were cut, fixed and stored as previously described (Cordell *et al.*, 1984).

Cell lines

The following cell lines were obtained from the Sir William Dunn School of Pathology, Oxford: K562 (erythroleukaemia), Hela (cervical epithelial carcinoma), U937 (malignant histiocytosis), HL60 (promyelocytic leukaemia), RVH421 (melanoma), A431 (vulval carcinoma), Daudi and Raji (Burkitt's lymphoma) and Molt-4 (T cell acute lymphoblastic leukaemia). The cell lines L428 (Hodgkin's disease), SU-DHL-1 (a T cell lymphoma with a 2;5 chromosomal translocation), KATO (gastric carcinoma) and umbilical endothelial cells were obtained from Dr V. Diehl, Dr M. L. Cleary, Prof P. M. Comoglio and the Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford, respectively.

All cell lines were cultured in RPMI 1640 and 10% fetal calf serum (FCS) (Gibco Bio-Cult, Paisley, UK) at 37°C. Cytocentrifuge preparations were made and treated as previously described. To promote their differentiation into macrophage cells, U937 cells were incubated with $1{\cdot}6 \times 10^{-8}$ M 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma Chemical Co.) in RPMI 1640 containing 10% FCS for 4 days.

Monoclonal reagents used

DAKO-CD19 (CD19, anti-B cell), DAKO-CD3 (CD3, anti-T cell) and DAKO-macrophage (CD68, anti-macrophage) were obtained from Dakopatts (Glostrup, Denmark). DJ130c (CD16, anti-granulocytes, monocytes and natural killer (NK) cells), 2LPM19c (CD11b, anti-monocytes and granulocytes), K92 (anti-keratin) and By114 (anti-granulocyte) were prepared in our own laboratory.

Immunocytochemical staining

The alkaline phosphatase–anti-alkaline phosphatase (APAAP) staining technique was performed as previously described (Cordell *et al.*, 1984; Erber *et al.*, 1984). The enzyme reaction was developed using naphthol AS-MX phosphate and Fast Red (TR-Salt) (Sigma Chemical Co.) as substrate.

Antibody blocking experiments were performed as follows. Sections of hairy cell spleen were incubated sequentially with antibody VMP55, biotinylated GHI/75 (prepared as described by Goding, 1983) and avidin conjugated to alkaline phosphatase (P365, Dakopatts). The binding of the biotinylated antibody was determined by developing the reaction with naphthol AS-MX phosphate and Fast Red substrate as above.

FACS analysis

Mononuclear tonsil cells were prepared as previously described and white cells were isolated from whole blood by red cell lysis using ammonium chloride (Pulford *et al.*, 1989). The resulting leucocytes were then incubated for 30 min at 4°C with MoAb in the form of undiluted supernatant. After washing, the cells were incubated for a further 30 min with fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse F(ab')2 immunoglobulin (F313) (Dakopatts). The cells were then washed, fixed in 1·5% formaldehyde in PBS and analysed by flow cytometry in a FACScan (Becton Dickinson). Lymphocyte, granulocyte and monocyte populations were analysed separately by gating on forward and side scatter measurements.

In some experiments cells were double labelled by incubation for 30 min at 4°C with biotinylated GHI/75 and either FITC-anti-CD3 (F818) or FITC-anti-CD19 (F768, Dakopatts). After washing, the samples were incubated with phycoerythrin-streptavidin (RPE), washed again, and then fixed and analysed by flow cytometry in the FACScan, using two-colour fluorescence. Double labelling was also carried out using FITC-anti-CD16 prepared as described by Goding (1983) and biotinylated GHI/75.

Immunoprecipitation and polyacrylamide gel electrophoresis

This was performed as described by Pulford *et al.* (1989). Briefly, antigen eluted from the lectin column was dialysed and 100 μg was labelled with 1 mCi ^{125}I (Amersham) in the presence of 20 μg Iodogen (Pierce Chemical Co., Rockford, IL). Labelled material was precleared using affinity purified rabbit anti-mouse immunoglobulin (Dakopatts) bound to *Staphylococcus aureus*. MoAbs were added to the precleared antigen and after 1 h rabbit anti-mouse immunoglobulin bound to *S. aureus* was added. After a further hour, the antigen was washed in buffer

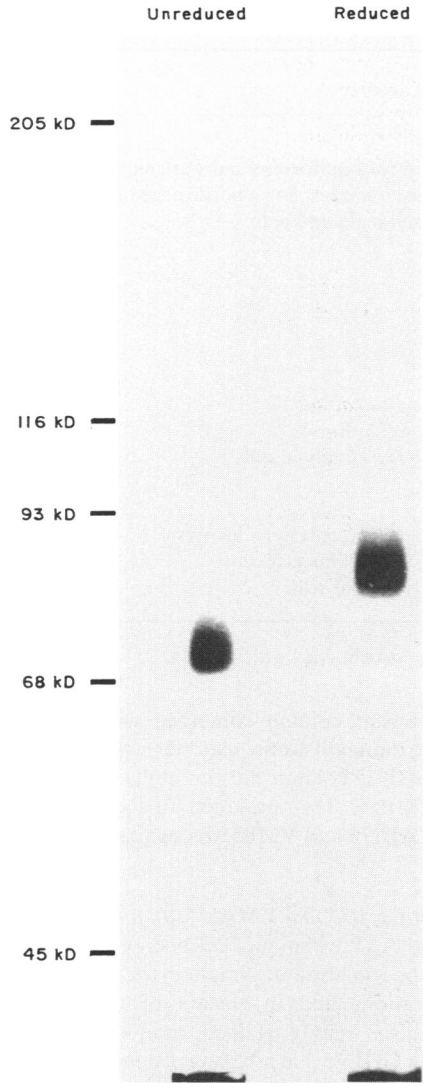

Fig. 1. Immunoprecipitation of the ^{125}I-labelled lentil lectin eluate of hairy cell leukaemia spleen. Antibody GHI/75 precipitates a prominent band of 72 kD molecular weight before reduction which increases to 83 kD after reduction. The same results are obtained with antibody VMP55 (not shown).

containing 1% NP40 and 500 mM NaCl. Immunoprecipitates were analysed by SDS–PAGE (Laemmli, 1970) followed by autoradiography of the dried gel using an enhancing screen.

Affinity purification

GHI/75 antibody was isolated from ascitic fluid by affinity chromatography and coupled to CNBr activated Sepharose (Pharmacia) at 5 mg/ml swollen gel according to the manufacturer's instructions. The lentil lectin affinity purified material was applied to the immunoabsorbant column and the column was washed with 250 mM NaCl and 1% NP40 in extraction buffer (see above). Specifically bound material was eluted with 50 mM glycine, 1% NP40 pH 2·2. Fractions containing antigen were identified by SDS–PAGE or Western blotting.

Western blotting

Dialysed antigen from the lectin column was run onto a 10% SDS polyacrylamide slab gel. Proteins were transferred electrophoretically overnight by Western blotting (Towbin, Staehlin & Gordon, 1979) onto nitrocellulose paper using a TRANSBLOT apparatus (Biorad, Richmond, CA). The paper was then stained using the APAAP method as described by Pulford *et al.* (1989).

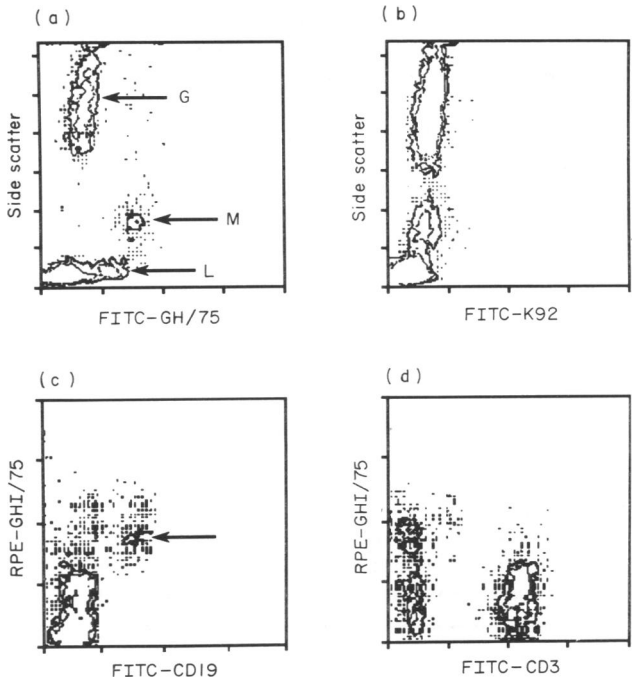

Fig. 2. FACS analysis of peripheral blood white cells. Using single labelling in (a) GHI/75 stains a small population of lymphocytes (L) and weakly stains monocytes (M). Granulocytes (G) remain unlabelled. No staining of any cell population can be seen with the irrelevant antibody K92 in (b). Two-colour immunofluorescence studies using lymphocyte gating were performed in order to determine the identity of the GHI/75 positive lymphocytes. From (c) it can be seen that all the FITC-CD19-positive B cells are also RPE-GHI/75-positive (arrowed). In contrast, in (d) no FITC-CD3-positive T cells are labelled by RPE-GHI/75.

RESULTS

Production of MoAbs GHI/75 and VMP55

Immunohistological screening on cryostat sections of hairy cell spleen identified two hybridoma cultures (one from each fusion) which produced antibody which gave strong staining of neoplastic cells. Very little staining was seen of cells in sections of human tonsil and the two cultures were cloned by limiting dilution to produce the clones designated GHI/75 and VMP55. Throughout the study very similar patterns of staining were seen with both GHI/75 and VMP55. Blocking experiments using VMP55 and biotinylated GHI/75 demonstrated complete blocking of the binding of the biotinylated antibody by VMP55, indicating that both antibodies recognized either the same epitope or epitopes in close proximity. However, VMP55 gave stronger labelling on tissue sections than GHI/75, while GHI/75 produced better results by flow cytometry.

Biochemical characterization

Both GHI/75 and VMP55 immunoprecipitated an antigen from a lysate of hairy cell leukaemia spleen with a molecular weight of 72 kD in the unreduced state and 83 kD when reduced (Fig. 1).

Table 1. Reactivity of antibodies GHI/75 and VMP55 with normal and reactive tissue*

Tissue	Labelling
Normal	
Tonsil	Strong staining of plasma cells. Weak labelling of mantle zones and scattered interfollicular cells
Thymus	Weak labelling of medullary and cortical macrophages. Some positive medullary lymphocytes
Spleen	Weak labelling of mantle zones, marginal zones and red pulp
Colon	Few positive lymphoid cells
Kidney	Scattered positive lymphoid cells
Liver	Weak positive Kupffer cells
Lung	Weak labelling of macrophages
Skin	Negative
Brain	Few positive lymphoid cells in grey matter
Placenta	Weak labelling of some macrophages
Heart	Negative
Prostate	Weak labelling of some macrophages and a few lymphoid cells
Reactive	
Reactive lymph node	Weak labelling of follicles, macrophages and some cells in paracortex
Dermatopathic lymphadenopathy	Weak labelling of germinal centres and some interfollicular cells
Sinus histiocytosis	Weak labelling of macrophages and some lymphoid cells.

* Both antibodies gave the same pattern of staining. However, stronger labelling was obtained with VMP55.

Table 2. Reactivity of antibodies GHI/75 and VMP55 on cell lines

	Monoclonal antibody	
Cell line	GHI/75	VMP55
Haematopoietic		
Myeloid		
U937	—	—
U937 (TPA-stimulated, 4 days)	(60%)±	(60%)±
HL60	—	—
K562	rare cells +	rare cells +
Lymphoid		
Molt 4	—	
Daudi	(90%)±	(90%)±
Raji	(10%)±	(90%)±
L428	—	—
SU-DHL-1	—	—
Karpas 299	—	—
Non-haematopoietic		
Kato II (carcinoma)	—	—
RVH421 (melanoma)	—	ND
Hela (carcinoma)	—	—
A431 (carcinoma)	—	—
MCF-7 (carcinoma)	—	ND
Umbilical endothelium	—	ND

±, weak staining. Percentages of stained cells are shown in parentheses.

* TPA, tetradecanoylphorbol-13-acetate.

After Western blotting, however, only the 72-kD form of the antigen was recognized by the two antibodies.

The antigen recognized by GHI/75 was easy to immunopurify and approximately 30 μg of antigen was obtained from 200 g of hairy cell spleen. Preclearing experiments using the immunoabsorbant column combined with Western blotting showed that the lentil lectin material which had been passed down the GHI/75 column did not contain the antigen recognized by VMP55. This provided further evidence that the antibodies GHI/75 and VMP55 recognize the same antigen.

Reactivity of GHI/75 and VMP55 with normal blood cells

Using the APAAP technique, antibodies GHI/75 and VMP55 stained a subpopulation of lymphocytes (3–9%) in cytocentrifuge preparations and in smears of normal blood. Both antibodies also weakly stained monocytes, VMP55 more strongly than GHI/75. No labelling of neutrophils was seen with either antibody. Comparable results were obtained by flow cytometry. The results of a typical experiment in which GHI/75 stained monocytes and a subpopulation of lymphocytes are shown in Fig. 2a. VMP55 gave identical results (not shown) and, as in blood smears, neither antibody stained granulocytes.

Double labelling experiments, using biotinylated GHI/75 in combination with FITC-anti-CD3 or FITC-anti-CD19, showed that all B cells (CD19-positive) were GHI/75-positive (Fig. 2c), whereas T cells (CD3-positive) were unstained by GHI/75 (Fig. 2d). Double labelling experiments using FITC-anti-CD16 showed that the GHI/75-positive cells were not NK cells.

Reactivity of GHI/75 and VMP55 with normal and reactive tissues

The reactivity of GHI/75 and VMP55 with normal and reactive tissues is summarized in Table 1. In tonsil, both antibodies stained plasma cells strongly but gave only very weak staining of mantle zones and scattered interfollicular cells. FACS analysis of a suspension of tonsil cells showed that 55% of cells were weakly labelled by GHI/75. Double labelling showed that they were all B cells (CD19-positive), no T cells (CD3-positive) being reactive with GHI/75. Weak staining of mantle zones was also found in the spleen, and both antibodies labelled marginal zones

Table 3. Reactivity of GHI/75 and VMP55 with leukaemias and lymphomas

Neoplasm	No. of cases	GHI/75-pos.	VMP55-pos.
Haematological			
Hairy cell leukaemia	12	12++	12+++
Chronic lymphocytic leukaemia	3	1±	1+
Prolymphocytic leukaemia	2	0/2	0/2
Plasmacytoma	1	1+++	1+++
Multiple myeloma	1	1++	1++
Acute myeloid leukaemia	3	0/3	0/3
Chronic myeloid leukaemia	5	0/5	0/5
NHL B cell			
Lymphoplasmacytic lymphoma	1	1++	1+++
Centroblastic/centrocytic lymphoma	7	1/7++	1/5++
Centroblastic lymphoma	7	1/7++	1/7++
Lymphoblastic lymphoma	2	0/2	0/2
Immunoblastic lymphoma	3	0/3	0/3
NHL T cell			
Lymphoblastic lymphoma	1	0/1	0/1
Pleomorphic lymphoma	1	0/1	0/1
Hodgkin's disease	9	0/9	0/9

The classification of lymphomas was carried out according to the Kiel classification.

and red pulp weakly. When tested on reactive lymphoid tissues both GHI/75 and VMP55 labelled B cell follicles weakly (Table 1). In addition, weak staining was also seen of numerous interfollicular cells.

GHI/75 and VMP55 labelled only scattered lymphoid cells in other normal haematopoietic and non-haematopoietic tissue studied. Both antibodies gave weak staining of some tissue macrophages.

Reactivity of GHI/75 and VMP55 on cell lines

The reactivity of GHI/75 and VMP55 on cell lines is summarized in Table 2. Both antibodies gave moderate labelling of 90% of Daudi cells, while GHI/75 and VMP55 stained 10% and 90% of the Raji cells, respectively. Both antibodies labelled approximately 60% of TPA-stimulated U937 cells. With the exception of a few scattered cells (<0·5%) in the K562 preparation, all other cell lines tested were unlabelled by antibodies GHI/75 and VMP55.

Reactivity of GHI/75 and VMP55 with leukaemias and lymphomas

The results are summarized in Table 3. MoAbs GHI/75 and VMP55 labelled neoplastic cells strongly in all cases of hairy cell leukaemia studied (Fig. 3a). Strong staining of neoplastic cells was also seen in cases of plasmacytoma, multiple myeloma and lymphoplasmacytic lymphoma, in keeping with the staining of plasma cells in tonsil sections. Apart from the single cases of chronic lymphocytic leukaemia, centroblastic and centroblastic/centrocytic lymphomas stained with GHI/75 and VMP55, all other B cell leukaemias and lymphomas were unreactive with GHI/75 and VMP55. No labelling of tumour cells was seen in T cell lymphomas or Hodgkin's disease.

DISCUSSION

The present paper describes the production, characterization and use of two new MoAbs (GHI/75 and VMP55) which recognize the same 72-kD antigen present on circulating B cells and monocytes. These two antibodies gave only weak staining of some B cells in normal lymphoid tissue, and when tested on a wide variety of tissues (both haematopoietic and non-haematopoietic) stained plasma cells strongly but gave only weak labelling of some macrophages and lymphoid cells. These findings are in keeping with the results obtained from studies on cultured cell lines, where both antibodies reacted only weakly with Daudi, Raji and TPA-stimulated U937 cells but not with epithelial or erythroid lines. All of the results given in this paper were obtained from cryostat material as neither VMP55 nor GHI/75 labelled cells in routinely fixed tissues.

In contrast to the weak labelling found with normal cells, antibodies GHI/75 and VMP55 gave very strong staining of the neoplastic cells in the 12 cases of hairy cell leukaemia studied. The cases of multiple myeloma, plasmacytoma and lymphoplasmacytic lymphoma tested were also strongly labelled by these antibodies. The finding that GHI/75 and VMP55 stained plasma cells in normal lymphoid tissue and the leukaemic cells in plasma cell neoplasms provides further evidence that hairy cell leukaemia is a tumour of B cells in the later stages of maturation (van den Oord, de Wolf-Peeters & Desmet, 1985; Anderson *et al.*, 1985). They may therefore be of practical importance in the diagnosis of hairy cell leukaemia.

There are other MoAbs to B cell-associated antigens which have not yet been assigned to CD groups whose reactivity resembles that of GHI/75 and VMP55. In 1984, Posnett *et al.* reported that antibody HC2 stained hairy cell leukaemia and reacted with a subpopulation of circulating B cells. The HC2 antigen is of a similar size (52–63 kD or 60–70 kD) (Posnett,

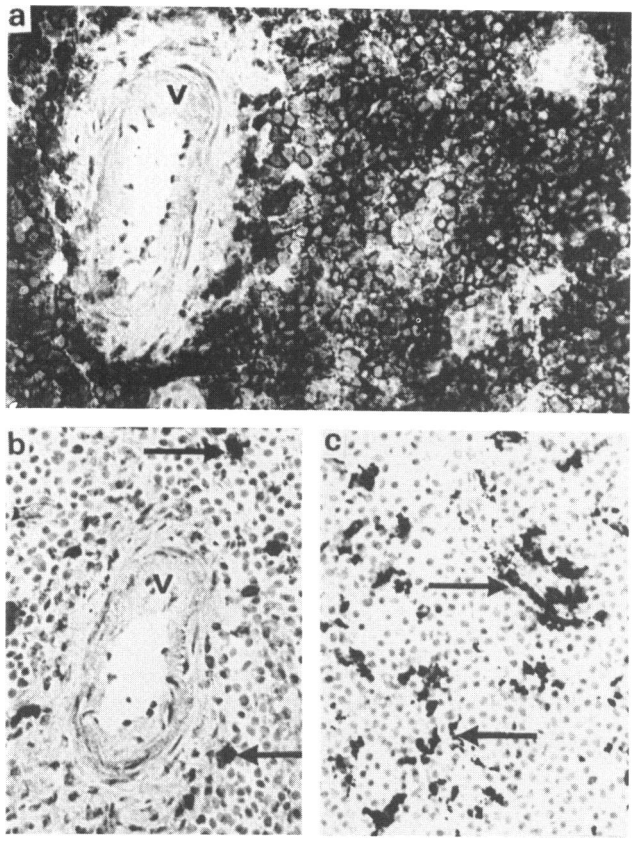

Fig. 3. APAAP staining of cryostat sections of hairy cell spleen. In (a) antibody GHI/75 gives very strong labelling of the neoplastic cells. (Note the unstained vessel, V.) (b) Staining of CD3-positive T cells (arrowed) among the hairy cells in an adjacent section. In (c) the hairy cells are negative for CD68, which is present on scattered macrophages (arrowed).

Duggan & McGrath, 1990) to the GHI/75 and VMP55 antigen (72, 83 kD) described in this study. However, later studies showed the HC2 antigen to be an antigen which appears on B cells, T cells and monocytes after activation (Posnett *et al.*, 1990). In addition, unlike GHI/75 and VMP55, antibody HC2 labels endothelium and does not stain all cases of hairy cell leukaemia (Falini *et al.*, 1985; Visser *et al.*, 1989).

Another marker found on circulating B cells, macrophages and hairy cell leukaemia (and also on intra-epithelial T lymphocytes, Schwarting *et al.*, 1990) is the molecule detected by antibodies B-Ly7, HML-1 and BerACT8 (Cerf-Bensussan *et al.*, 1987; Visser *et al.*, 1989; Kruschwitz *et al.*, 1990; Möller, Mielke and Moldenhauer, 1990; Pallesen & Hamilton-Dutoit, 1990). However, GHI/75 and VMP55 recognize an antigen of 83 kD (reduced), whereas antibodies B-Ly7, HML-1 and BerACT8 have all been found to recognize a trimeric antigen comprising polypeptides of 170, 140 and 110 kD (Schwarting *et al.*, 1990; Kruschwitz *et al.*, 1990).

One other previously reported antibody, B5, resembles GHI/75 and VMP55 (Freedman *et al.*, 1985). This reagent was initially described as staining only activated B cells, and was subsequently reported to label circulating B cells and 30% of monocytes (Pezzutto *et al.*, 1989). Like GHI/75 and VMP55, antibody B5 stains Raji and Daudi cells. One further similarity between the three antibodies is the size of their target antigens, since B5 recognizes 75-kD and 67-kD bands under reducing and non-reducing conditions respectively (Freedman *et al.*, 1985), values which are comparable to those of 83 kD and 72 kD for antibodies GHI/75 and VMP55.

Antibody B5 was submitted to the Fourth International Workshop on Human Leucocyte Differentiation Antigens (1989) and was not clustered with any other reagents. It would therefore be interesting to see in the future whether GHI/75 and VMP55 recognize the same molecule and can therefore be used to establish the existence of a new B cell-associated molecule with a CD designation. In addition, the fact that the target molecule is easily purified from extracts of human spleen by immuno-affinity chromatography (see Results) means that the sequencing of this protein and isolation of the gene which encodes it are technically feasible.

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