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Technical Advance

New Monoclonal Antibodies to the T Cell Antigens CD4 and CD8

Production and Characterization in Formalin-Fixed Paraffin-Embedded Tissue

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We have generated a recombinant protein representing part of the CD4 molecule and a peptide representing an epitope of predicted high antigenicity on the CD8 molecule and employed these to generate mouse monoclonal antibodies using standard hybridoma protocols. The extracellular domain of the CD4 molecule was obtained by reverse transcription of mRNA from peripheral blood lymphocytes followed by polymerase chain reaction. The amplified gene fragment was cloned into an expression vector to allow a histidine-tagged fusion protein to be produced in *Escherichia coli*. Purified fusion protein was used to immunize mice. The CD8 monoclonal antibody was raised against a peptide consisting of 13 amino acids within the carboxyl-terminal region of the CD8 cytoplasmic domain. The antibodies showed appropriate reactivity on Western blotting. By heat pretreatment, these antibodies have been shown to be highly effective on paraffin-embedded tissue. In normal lymphoid tissue, the expected distribution of CD4 and CD8 lymphocytes was observed. In a series of 16 T cell lymphomas and B cell lymphomas, immunostaining results were compared with those obtained using reagents effective only in frozen tissue. A high degree of correlation was observed. These results suggest that NCL-CD4 and NCL-CD8 may be of value in the characterization of T cell disorders. *(Am J Pathol 1998, 152:1421–1426)*

The accurate diagnosis of malignant lymphomas often requires the assessment of the phenotype of the lymphoid cells.[1,2] A wide range of antibodies is available for use on frozen sections, but because some antigens become denatured during fixation and processing, a smaller range is available for use on formalin-fixed, paraffin-processed tissues. Paraffin sections provide better tissue morphology than frozen sections and, because of the better cytological and architectural preservation, provide for better assessment of antigen localization. In many cases, frozen material is not available because of the size of the biopsy, fears of infection, or local surgical practice. Sometimes tissue is not frozen because lymphoma is not suspected or because the laboratory does not have appropriate facilities. Phenotypic characterization would be enhanced by the availability of more antibodies effective in paraffin-embedded tissue.

T cells express several specific antigens. CD4 is a marker of helper-inducer T cell populations; it is expressed on approximately two-thirds of peripheral blood T cells. It is also expressed on cells of histiocytic lineage. The CD4 molecule has an extracellular domain containing four IgSF domains.[3] The CD4 molecule is involved in the recognition of foreign antigens in association with major histocompatibility complex (MHC) class II antigens.[4] In addition, CD4 is a receptor for the human immunodeficiency virus. CD8 is expressed on cytotoxic suppressor T cells. It is expressed as a heterodimer of CD8α and CD8β.[3,4] CD8 is expressed on approximately one-third of peripheral blood T cells (the CD4-negative cells). CD8 has also been detected at a low level on some natural killer (NK) cells. CD8 acts as a co-receptor with MHC class I antigens in the recognition of antigens.[4]

We have employed synthetic antigens (peptides and recombinant proteins) to generate monoclonal antibodies

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that recognize CD4 and CD8. These antibodies have been evaluated in a range of normal tissues and T and B cell lymphomas.

Materials and Methods

Tissues

Sixteen cases of peripheral T cell lymphomas, six cases of peripheral B cell lymphoma, and one case of Hodgkin's disease were obtained from the files of the University of Edinburgh Department of Pathology. The T cell lymphomas included three cases of enteropathy-associated T cell lymphoma; the other cases were (using the Kiel classification) five cases of pleomorphic large T cell lymphoma, four cases of T immunoblastic lymphoma, one case of T zone lymphoma, two cases of Sézary syndrome, and one case of angio-immunoblastic lymphoma. Under the REAL classification they would be designated as 10 cases of peripheral T cell lymphoma, unspecified, two cases of Sézary syndrome, and one case of angio-immunoblastic lymphoma. The B cell lymphomas were all centroblastic lymphoma (Kiel)/diffuse large B cell lymphoma (REAL). All of the cases had previously been assessed (by A.S. Krajewski) using a panel of antibodies on frozen tissue sections and have been the subject of previous publications.[5,6] For assessment of reactivity of the antibodies in normal lymphoid and other tissues, paraffin blocks from tonsil, thymus, small and large intestine, liver, heart, kidney, and lung were retrieved from the files of the Department of Pathology at the Royal Victoria Infirmary, Newcastle upon Tyne. Paraffin-embedded blocks of all the tissues were prepared after fixation in 10% neutral buffered formalin for approximately 24 hours.

Production of Immunogens

Recombinant Protein CD4

Peripheral blood lymphocyte RNA was purified (as described in Chomczynski et al[7] and used as template for a reverse transcription reaction using a specific CD4 primer (CD4-R 5′AAGGAT CCT CAC ATT GGC TGC ACC GGG GTG 3′). Reaction conditions were as described in Steward et al.[8] Half of the reverse transcription mix was then used in a polymerase chain reaction (PCR) after the addition of a second primer (CD4-F 5′ GGG ATC CGA AAG TGG TGC GCA A 3′) and appropriate adjustment of conditions. The efficacy of PCR was determined by agarose gel electrophoresis and the product cloned into pUC57/T (MBI Fermentas, Sunderland, UK). Recombinant clones were identified and characterized, and the CD4 cDNA was subcloned into pET 15b (Novagen, Cramlington, UK). Low levels of insoluble protein were observed upon induction with 1 mmol/L isopropyl thiogalactoside in *Escherichia coli* BL21 (DE3)pLysS. Inclusion bodies were solubilized in 8 mmol/L urea/10 mmol/L 2-mercaptoethanol and refolded by repeated dilution with changes of 10 mmol/L Tris, pH 8.0. Refolded CD4 was then purified by column chromatography on His-band resin (Novagen) and was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis before immunization of female BALB/c mice.

Peptide for CD8

The peptide corresponding to the 13 carboxyl-terminal amino acids of the cytoplasmic domain of CD8 was synthesized (CKSGDKPSLSARYV).[9] The terminal cysteine was added to allow the linkage of the peptide to carrier proteins bovine serum albumin and keyhole limpet hemocyanin. The peptide was purified by high-pressure liquid chromatography and conjugated via the terminal cysteine.

Hybridoma Production and Screening

Initial immunizations of Balb/c mice were in Freund's complete adjuvant, followed by Freund's incomplete adjuvant, both administered subcutaneously. Subsequent immunizations were in phosphate-buffered saline and were administered intraperitoneally. Four days after the final intravenous boost, the spleen was removed and a cell suspension was produced. The spleen cells were then fused, using polyethylene glycol, with NS1/1-Ag4-1 cells and plated out into hypoxanthine aminopterin thymidine (HAT)-selective growth medium. The cultures were incubated for 10 to 14 days or until colony growth was apparent. Supernatant was removed from each well and assayed by enzyme-linked immunosorbent assay (ELISA) for reactivity against the appropriate recombinant CD4 protein or CD8 peptide conjugate. Wells exhibiting anti-CD4 or -CD8 activity were examined for colony growth and, where possible, individual colonies were transferred to separate wells in 24-well growth plates. After an additional incubation period, supernatant from each growth well was assayed by ELISA. Supernatant that gave positive ELISA was tested immunohistochemically using an indirect immunoperoxidase technique on heat-treated paraffin sections of normal human tonsil. Colonies demonstrating positive immunohistochemical staining were cloned by limiting dilution a minimum of four times. A total of 2860 hybridomas were produced for CD4; of these, 373 were ELISA positive. Fifty gave positive staining on paraffin sections, and strong staining was seen with two. The colony producing the most intense staining was selected. For CD8, 5470 hybridomas were produced. Seventeen were ELISA positive and two of these gave successful results with paraffin sections. Again, the colony producing the most intense staining was selected.

Western Blotting

Extracts of peripheral blood lymphocytes and Sup-T (T lymphoid cell line) were fractionated on 10% SDS-PAGE, and the proteins were transferred to nitrocellulose as described previously.[8] After blocking, the nitrocellulose

Table 1. Antibodies Used for Assessment of the Series of Malignant Lymphomas

	Designation	Clone	Source
Frozen sections	CD3	Leu 4	Becton Dickinson
	CD3	OKT3	Ortho Diagnostics
	CD4	T3-10	Dako
	CD8	DK25	Dako
	CD2	T9-10	Dako
	CD19	HD37	Dako
	CD20	B1	Ortho Diagnostics
	CD22	Dako-B	Dako
Paraffin sections	CD3*	Polyclonal	Dako
	NCL-CD3	PS1	Novocastra
	CD4	1F6	This study
	CD8	4B11	This study

*Rabbit polyclonal; all the rest are monoclonal.

was cut into two strips; that containing the peripheral blood lymphocyte extract was placed in a 1:10 dilution of NCL-CD8-4B11, and the strip containing the Sup-T extract was placed in a 1:10 dilution of NCL-CD4-1F6. After a 1-hour incubation, the strips were transferred to a 1:1000 dilution of alkaline-phosphatase-conjugated rabbit anti-mouse antiserum (Dako, High Wycombe, UK) and incubated for another hour. The blot was developed with 5-bromo-4-chloro-indolylphosphate/nitroblue tetrazolium (NBT/BCIP) until bands had developed and stopped by brief immersion in 10% acetic acid. The molecular weight of the visualized products was determined in relation to a series of standard molecular weight markers (Bio-Rad, Hemel Hempstead, UK).

Immunohistochemistry

The monoclonal antibodies used are shown in Table 1. An indirect immunoperoxidase technique was used for frozen section immunohistochemistry.[10] For paraffin section immunohistochemistry, 5-μm sections were cut from each paraffin block and mounted onto slides coated with Vectabond (Vector Laboratories, Burlingame, CA) and dried overnight at 39°C. After 60 minutes of drying at 60°C, the sections were dewaxed, rehydrated, and treated with H_2O_2/methanol for 10 minutes. The sections were then washed in running tap water for 5 minutes. Before addition of the primary antibody supernatant, one of two types of heat treatment was applied, either microwaves[11,12] or the high-temperature antigen unmasking technique.[13] For either method, the dewaxed sections were treated immersed in 1 mmol/L EDTA at pH 8.0. The sections were then covered with normal rabbit serum (NRS) that had been diluted 1 in 5 with Tris-buffered saline (TBS) and incubated for 10 minutes at room temperature in a humidified chamber. After removal of excess serum, appropriately diluted primary antibody was added to the sections and incubated at 25°C for 60 minutes. NCL-CD4-1FG and NCL-CD8-4B11 were diluted 1 in 20 with NRS. Supernatants were applied neat during initial screenings. As a negative control, diluted normal goat serum was added to a section and incubated as described. Sections were washed in TBS for 10 minutes, changing the wash after 5 minutes. They were then incubated for 30 minutes with biotinylated rabbit anti-mouse immunoglobulins (NCL-GAMP, Novocastra Laboratories, Newcastle upon Tyne, UK) diluted 1 in 500 with NRS. The sections were incubated for 30 minutes with avidin-peroxidase at 25°C. After an additional two 5-minute washes in TBS, enzyme activity was revealed using a diaminobenzidine/H_2O_2 substrate.

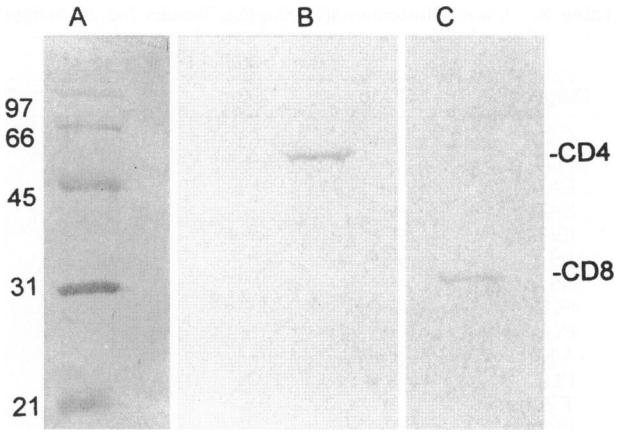

Figure 1. Detection of CD4 and CD8 proteins in cell extracts by Western blotting using NCL-CD4-1F6 and NCL-CD8-4B11. **Lane A**, molecular weight markers, sizes indicated ($\times 10^{-3}$ kd); **Lane B**, Sup-T cell lysate probed with NCL-CD4-1F6; the single clear band at approximately 55 kd is indicated; **Lane C**, peripheral blood lymphocyte probed with NCL-CD8-4B11; the single clear band at approximately 32 kd is indicated.

Results

Monoclonal Antibody Production and Evaluation

Monoclonal antibodies NCL-CD4-1FG and NCL-CD8-4B11 have been generated and evaluated as follows.

Western Blotting

NCL-CD4-1FG showed a band of reactivity at 55 kd (Figure 1). This value is similar to that identified by Secchiero et al[14] using anti-CD4 monoclonal cel 3A. NCL-CD8-4B11 stained a band at molecular weight 32 kd, which is in agreement with previous Western blotting results obtained by Mason et al[9] using a panel of anti-CD8 antibodies developed in their laboratories.

Immunohistochemical Staining of Normal Tissues

In normal human tonsil, the distribution patterns of CD4-positive cells and CD8-positive cells were similar. Large numbers of positively staining lymphocytes were present within the paracortex; occasional positive cells were also identified within germinal centers and within the investing squamous epithelium. In other tissues, only lymphoid cells and cells of histiocyte lineage showed positive staining for CD4 and CD8. Positive staining of the cells was not observed when the antigen retrieval techniques were not used.

Table 2. Immunohistochemical Staining Results for 23 Malignant Lymphomas Stained for T Cell Markers

	Paraffin sections				Frozen sections				
Diagnosis	CD3p	CD3m	CD4	CD8	CD3	CD4	CD8	Pan B	CD2
EATL	++	++	−	+++	++	−	−	−	−
PLC	+	+	+	−	++	++	−	−	++
EATL	++	++	+	−	+	−	−	−	++
EATL	−	−	−	−	+	−	−	−	−
IB(T)	++	+++	±	++	++	++	+	−	++
SS	++	+	+	−	++	++	−	−	++
SS	+	++	+	−	++	++	−	−	++
PLC	−	−	+	−	−	++	−	−	++
PLC	−	−	+	−	−	++	−	−	++
AIL	++	++	+	++	++	+	+	−	++
PLC	++	+	+	−	−	++	−	−	−
T Zone	+	+	+	−	++	++	−	−	++
IB(T)	+	+	+	−	++	++	−	−	++
PLC	++	++	++	−	++	++	−	−	++
IB(T)	++	++	+	−	++	++	−	−	++
IB(T)	+++	+++	+	−	++	−	++	−	−
HD	−	−	−	−	−	−	−	−	
CB	−	+	−	−	−	ND	ND	++	
CB	−	−	−	−				++	
CB	−	−	−	−	−	ND	ND	++	
CB	−	−	−	−	−	ND	ND	++	
CB	−	−	−	−	−	−	−	++	
CB	−	−	−	−	−	−	−	++	

EATL, enteropathy-associated T cell lymphoma; PLC, pleomorphic large-cell lymphoma; IB(T), T cell immunoblastic lymphoma; SS, Sézary syndrome; AIL, angio-immunoblastic T cell lymphoma; T zone, T zone lymphoma; HD, Hodgkin's disease; CB, B cell centroblastic lymphoma. ND, not done. The pan-B cell markers were CD19, CD20, and CD22.

Immunohistochemical Staining in Lymphoid Neoplasms

Sixteen cases of peripheral T cell lymphoma were examined. The results are shown in Table 2. The staining pattern of CD3, CD4, and CD8 was cytoplasmic with membrane accentuation. Typical staining results from this series and other cases are shown in Figure 2.

In 12 of the 16 cases, the neoplastic cells were positive for CD4 on frozen sections. All of these cases were positive for CD4 on paraffin sections. In 10 of these cases, the neoplastic cells were negative for CD8, both on frozen sections and on paraffin sections. Two cases that showed CD4-positive staining of the neoplastic cells (both on frozen sections and paraffin sections) also showed positive staining of the neoplastic cells with CD8, using both frozen sections and paraffin sections. These were one case of immunoblastic T cell lymphoma/peripheral T cell lymphoma, unspecified, and one case of angio-immunoblastic lymphoma.

In one case, the neoplastic cells were positive for CD8 but negative for both CD4 antibodies. The positive staining for CD8 was seen on both frozen and paraffin sections. This was a small, partly necrotic sample of a T cell immunoblastic lymphoma/peripheral T cell lymphoma, unspecified.

Three cases gave negative staining of the tumor cells for both CD4 and CD8 on the frozen sections. They were all cases of enteropathy-associated T cell lymphoma. Two of these cases were positive for CD3 on both frozen and paraffin sections; on paraffin sections, one case was CD8 positive and the other weakly positive for CD4. The third case was weakly positive for CD3 on frozen sections and negative for CD3, CD4, and CD8 on paraffin sections.

Six cases of centroblastic/diffuse large B cell lymphoma were examined. In all cases, the neoplastic cells were positive for the pan-B cell markers (CD19, CD20, and CD22). One case showed weak positivity of the tumor cells with the monoclonal CD3 antibody on paraffin sections. All of the cases were negative for all of the other CD3, CD4, and CD8 markers. One case of Hodgkin's disease was included in the study; the neoplastic cells were negative for all of the markers examined.

Discussion

We have compared the results using our antibodies with the established markers to CD4 and CD8 used on frozen sections of the same tissue. In all cases in which the neoplastic cells were positive for either CD4 or CD8 or both on frozen sections, the paraffin section staining gave the same result. One case was negative for all four antibodies. In only 2 cases of T cell lymphoma from our series of 16 cases were the staining results different on paraffin and frozen sections. One of these cases was CD8 positive on paraffin sections only; the other was weakly CD4 positive on paraffin sections only. Both were cases of enteropathy-associated T cell lymphoma. No neoplastic B cells were stained by any antibody to CD4 or CD8. The high degree of correlation between the paraffin section and frozen section antibodies supports the potential value of these paraffin section antibodies.

Most peripheral T cell lymphomas (unspecified) and most chronic T lymphocytic leukemias have CD4-positive

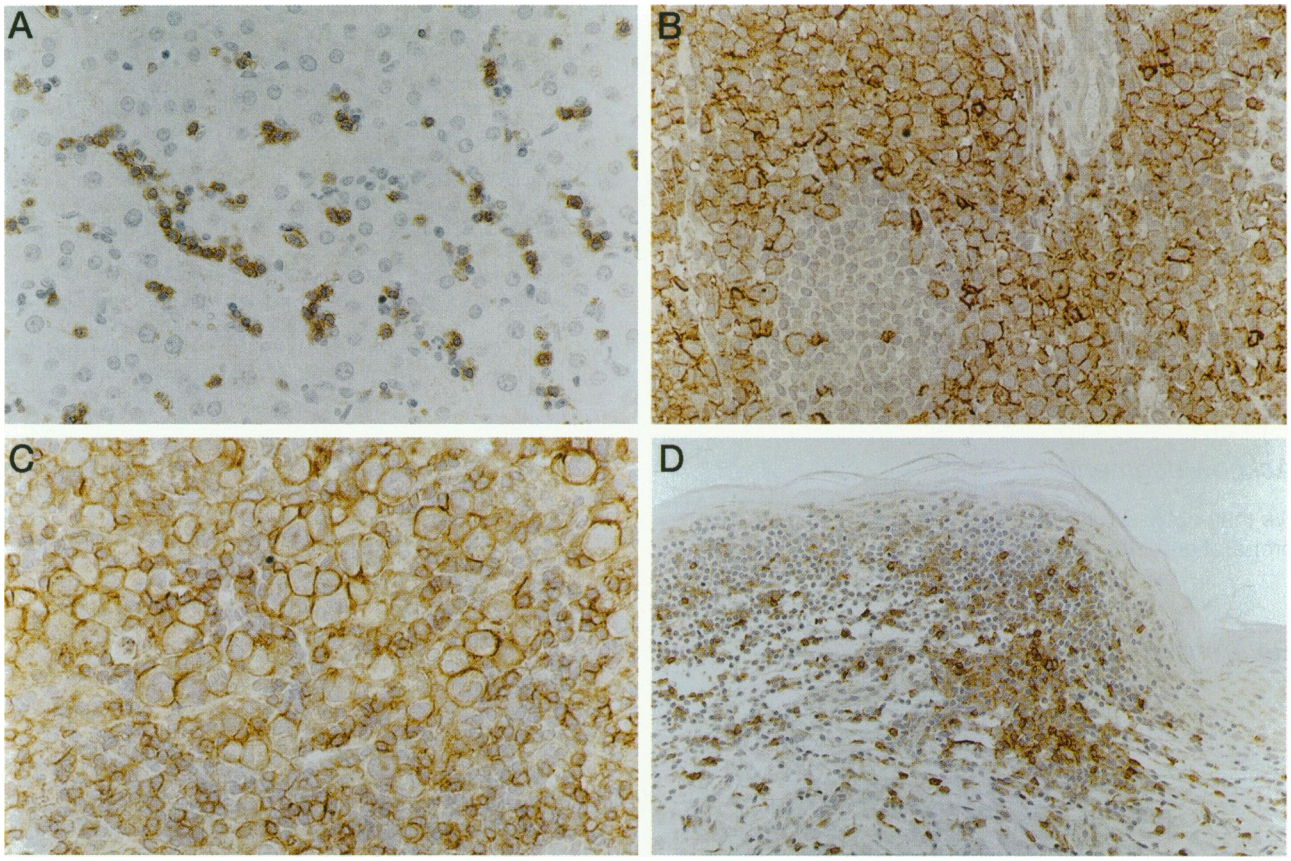

Figure 2. A: Liver, hepatosplenic γ-δ T cell lymphoma. Immunostain is NCL-CD8. The tumor cells within the hepatic sinusoids show clear and intense labeling. **B:** Lymph node, peripheral T cell lymphoma. Immunostain is NCL-CD8. Tumor cells show intense membrane and rather weaker cytoplasmic staining. **C:** Peripheral T cell lymphoma forming a nasal mass. Immunostain is NCL-CD4. Tumor cells show intense membrane and rather weaker cytoplasmic staining. **D:** Skin, mycosis fungoides. Immunostain is NCL-CD4. The dermal lymphoid infiltrate shows immunostaining of the majority of the cells for NCL-CD4.

neoplastic cells.[1] Classically, large granular lymphocytic leukaemias, hepatosplenic T cell lymphoma and subcutaneous paniculitic T cell lymphoma are CD8 positive.[2] The identification of either CD4 or CD8 staining within the neoplastic cells will therefore aid the correct diagnosis of these tumors. Most cases of mycosis fungoides contain CD4-positive neoplastic cells with CD8-positive reactive cells.[15]

The clear identification of the neoplastic cells, the localization of the positive staining within these cells, and the correlation of morphology with phenotype are crucial if diagnostic use is to be made of this form of immunophenotypic analysis. The superior morphology and preservation of cell detail achieved in paraffin sections rather than frozen sections is an obvious advantage.

Some peripheral T cell lymphomas show aberrant expression of T-cell-associated antigens.[1,5,16] The lack of expression of one or more pan-T cell marker by T cell lymphomas is frequently observed.[8,16] The simultaneous expression of both CD4 and CD8, or loss of expression of both these antigens, may be seen. The demonstration of aberrant T cell phenotypes may be a useful supplement for the diagnosis of T cell malignancies.[16] T-lymphoblastic lymphoma is often positive or negative for both CD4 and CD8.[2] Some T cell markers are not lineage specific and will stain cells that are not T cells.[1,8] These findings emphasize the need for a panel of antibodies to characterize T cells. Our antibodies to CD4 and CD8 will broaden the range available for such panels and help to increase their sensitivity and specificity. It is important to note that CD4 expression is not confined to T cells; for example, expression of CD4 is reported in cells of histiocyte lineage, Langerhans cells, and the cells of Langerhans cell histiocytosis.[17,18] We have observed strong labeling for one case of Langerhans cell histiocytosis (data not shown).

Immunophenotypic analysis of T cell subsets is potentially useful[19,20] for the diagnosis and investigation of non-neoplastic reactive and inflammatory conditions. For example, our understanding of graft-*versus*-host disease may be advanced by studying the proportions of CD4-positive and CD8-positive T cells within target organs. When an antibody to CD4 was not available for paraffin-processed tissues, this could be done using frozen sections,[19] but in cases in which frozen material was not available, the number of CD4-positive cells in the target organs had to be estimated indirectly from CD3 and CD8 staining.[20] There is a wide interest in studying the inflammatory response to tumors, such as breast carcinomas,[21] and the antibodies described here may prove of value in this field.

Other authors have described the use of a CD8 peptide sequence to make antibodies suitable for use in paraffin sections.[9] We describe the production of another

antibody to CD8 for paraffin sections. The CD8 antibodies produced by both groups were raised to an identical peptide using similar hybridoma methods to produce monoclonal antibodies. It is likely that their reactivity in tissue sections will be similar. We believe we are the first group to successfully make an antibody to CD4 for paraffin section use. These antibodies can be used as a pair, exploiting their cell-type-specific expression and aberrant expression to provide greater diagnostic information. They may prove to be a useful addition to the current panel of antibodies used in the characterization and study of lymphomas and lymphoid cells in routinely processed tissue.

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