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Technical report

A new monoclonal antibody to human subcapsular thymic epithelial cells

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Summary. A monoclonal antibody, termed K-20, was generated against an anaplastic thymic carcinoma cell line, Ty-82. Subcapsular thymic epithelial cells of the thymus and blood vessels in various organs were shown to react with the K-20 monoclonal antibody by immunohistochemical staining. Immunofluorescent study revealed that various haematopoietic fresh cells and cell lines did not show any significant reactivity with K-20, except for one Epstein-Barr-virus-carrying lymphoma cell line (SP-50B). Western immunoblotting and affinity purification procedure revealed that K-20 was directed to a protein with a molecular weight of 28 kDa. K-20 is unique in its restrictive reactivity with human subcapsular thymic epithelial cells.

Key words: Monoclonal antibody – Thymic epithelial cells – Thymic cancer – K-20 – Immunohistochemistry

Introduction

The thymic epithelial cell is a major component of the thymic microenvironment. It plays an important role in T-cell development acting through direct interaction with thymocytes and boy production of thymic factors (Goldstein et al. 1966, 1977; Low et al. 1981). Recent study based on ultrastructural and immunohistochemical analysis has indicated the heterogeneity of thymic epithelial cells (Van de Wijngaert et al. 1984; Kampinga et al. 1989). This heterogeneity is essential for the development of T-cells, following various steps in thymic environment. Thus, the preparation of monoclonal antibodies to subtypes of thymic epithelial cells may be a useful approach for the better understanding of this microenvironment.

The subcapsular cortical region is known to be the site where immature thymocytes proliferate and differentiate. This region represents the site to which thymocyte precursors first migrate on entering the thymus. Thus, thymic epithelial cells in the subcapsular cortex may be important in the induction of early lymphocyte differentiation (Ritter et al. 1981).

Recently, we have established a cell line, Ty-82, from a patient with thymic carcinoma, which is a rare malignant neoplasm of thymic epithelial cell origin (Hartmann et al. 1990). In the present study, we generated a monoclonal antibody, designated K-20, to Ty-82 cells in order to detect the thymic epithelial cell antigen. K-20 immunostained subcapsular thymic epithelial cells almost exclusively and vascular endothelial cells in various tissues.

Materials and methods

Established human haematopoietic cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). A squamous carcinoma cell line LC-1 sq and an anaplastic thyroid cancer cell line TCO-1 were maintained in Dulbecco's modification of Eagle's medium (D-MEM) supplemented with 10% FCS. Different types of normal peripheral blood cells were obtained from ten healthy volunteers using Mono-Poly Resolving Medium (Flow laboratories, Irvine, Calif.) (Ferrante and Thong 1978). Non-neoplastic bone marrow cells were obtained from three patients with malignant lymphoma in which no abnormal cells were detectable. Various human normal tissues were obtained fresh from cadavers at our hospital. Thymuses were obtained from four neonatal cadavers, aged from 1 to 5 weeks. The frozen sections of normal tissues were fixed in acetone at 4° C for 5 min and kept in −70° C until use.

The detailed procedures for the produchain of the monoclonal antibodies have been described (Takeuchi et al. 1988). Briefly, 1×10^6 intact Ty-82 cells were intraperitoneally injected into BALB/c mice. After the third immunization, the mice were sacrificed under ether anaesthesia and splenocytes were fused with partner cells, P3/NS1/1-Ag4-1 (NS-1). After the third cloning by limiting dilution, hybridomas were established. The isotype of a monoclonal antibody was determined by the double immunodiffusion test as described previously (Takeuchi et al. 1988). Monoclonal antibodies were purified from culture supernatants using protein-A Sepharose CL-4B (Pharmacia, Uppsala, Sweden).

Isotype matching monoclonal antibody against an unknown antigen was used as a negative control. A preliminary test revealed that this antibody did not show any significant reactivity with var-

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ious human tissues. Anti-cytokeratins nos. 1, 5, 10, 11 and no. 8 were purchased from Enzo Diagnostics (New York, USA). Anti-HLA-DR (L243) and CD57 (Leu7) were purchased from Becton-Dickinson (Mountainview, Calif., USA). Anti-p19 were purchased from Cellular Products (Buffalo, N.Y., USA).

Indirect immunofluorescent staining was carried out as described previously (Takeuchi et al. 1988). Sections of various tissues were stained by the indirect immunoperoxidase method as previously reported (Takeuchi et al. 1988). Briefly, endogenous peroxidase was inhibited by methanol containing 0.3% hydrogen peroxide for 20 min. Sections were then incubated with normal goat serum. These sections were incubated for 1 h at room temperature with monoclonal antibodies (20 μg/ml), followed by incubation for 1 h with diluted goat anti-mouse Ig conjugated with peroxidase (Dakopatts, Copenhagen, Denmark). The peroxidase was developed with Diaminobenzidine (DAB)-hydrogen peroxide.

Cell membrane extracts of Ty-82 cells were prepared according to the method of Seon et al. (Seon et al. 1981; Matsuzaki et al. 1987). Cell membranes were prepared from Ty-82 cells and solubilized by sodium deoxycholate (DOC) treatment. A neonatal thymus was homogenized with 1% NP40-Tris HCl buffer (pH 7.4). All procedures were performed in a cold room and in the presence of 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 0.03% sodium azide.

Western immunoblotting was carried out according to the method of Towbin et al. (1979). Samples in the presence or absence of 2-mercaptoethanol were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene difluoride membranes (Millipore, Bedford, Mass.). After blocking, the membranes were incubated with monoclonal antibodies (20 μg/ml) overnight at 4° C. After washing, membranes were incubated with diluted anti-mouse Ig conjugated with peroxidase (Dakopatts) for 2 h at room temperature. The peroxidase was detected in DAB-hydrogen peroxide.

The purification of the antigen was performed by immunoaffinity chromatography. 0.5% DOC extracts of 5×10^9 Ty-82 cells were first applied to a Sephadex G-75 column (Pharmacia). K-20 antigen-rich fractions were detected by dot blotting, then applied to control IgG immobilized on Affi-Gel Hz hydrazide gel (Bio-Rad, Richmond, Calif.). The breakthrough fraction was collected and applied to the immobilized K-20 Affi-Gel Hz column. Elution was done with 0.5% DOC buffer containing 50 mM diethylamine (pH 11.0). All procedures were performed in a cold room and in the presence of 0.03% sodium azide and 0.5 mM PMSF.

Results

A monoclonal antibody, designated K-20, was selected and established after the third cloning. Isotype analysis revealed that K-20 was of an IgG1 heavy chain subclass.

K-20 did not show any significant reactivity with haematopoietic cell lines tested, except for a SP-50B cell line by indirect immunofluorescent study. Neither peripheral mononuclear cells, granulocytes, red blood cells nor normal bone marrow cells were stained with K-20. In contrast, Ty-82 cells showed a strong reactivity with K-20. These results are summarized in Tables 1 and 2.

Subcapsular thymic epithelial cells from four individual neonates demonstrated an intense staining with K-20. The antibody also immunostained the inner cortex faintly in a dendric pattern. However, neither epithelial cells in the medulla nor the Hassall's corpuscles demonstrated positive staining with K-20 (Fig. 1). Anti-cytokeratin nos. 1, 5, 10, 11 antibody also immunostained these K-20-positive cells, although it also demonstrated reactivity with the medullary thymic epithelial cells and Hassall's corpuscles. Anti-cytokeratin no. 8 stained some thymic epithelial cells in the medulla and some Hassall's corpuscles. Anti-HLA-DR strongly immunostained thymic epithelial cells, as shown in Fig. 1. Anti-p19 and -CD57 also demonstrated positive staining with the subcapsular cortex.

As summarized in Table 3, blood vessels in various tissues demonstrated a positive reactivity with K-20. However, nephrons of the kidneys lacked reactivity with K-20.

A single band corresponding to a molecular weight of 28 kDa was detected in the lysates of a neonatal thymus and Ty-82 cells under a non-reduced condition by the immunoblotting method (Fig. 2). No bands were observed in the presence of 2-mercaptoethanol. K-20 antigen was partially purified by immunoaffinity chromatography and applied to SDS-PAGE. A major band with a molecular weight of 28 kDa was detected in the presence or absence of 2-mercaptoethanol. This purified protein appeared to be reactive with K-20 antibody by immunoblotting under a non-reduced condition (Fig. 3).

Table 1. Reactivity of K-20 against established cell lines

Cell lines		% of positive cells
Myeloid	PL-21	0
	KCL-22	0
B-cell	SP-50B	90
	SP-53	0
	RCK-8	0
	BALL-1	0
	Raji	0
	EB virus-immortalized cells ($n=5$)	0
T-cell	TALL-1	0
	MT-1	0
	ATL-1K	0
Non-T, non-B		
	NALM-18	0
	KEN-L-1	0
Lung squamous carcinoma		
	LC-1 sq	0
Thyroid anaplastic cell cancer		
	TCO-1	0

Table 2. Reactivity of K-20 with normal haematopoietic cells

Cells	Reactivity[a]
Peripheral blood	
Mononuclear cells	0/10
Granulocytes	0/10
Erythrocytes	0/10
Bone marrow cells	0/3

[a] Number of reactive specimens per number of specimens tested

Discussion

In the present study, we generated a monoclonal antibody, termed K-20, against a thymic carcinoma cell line,

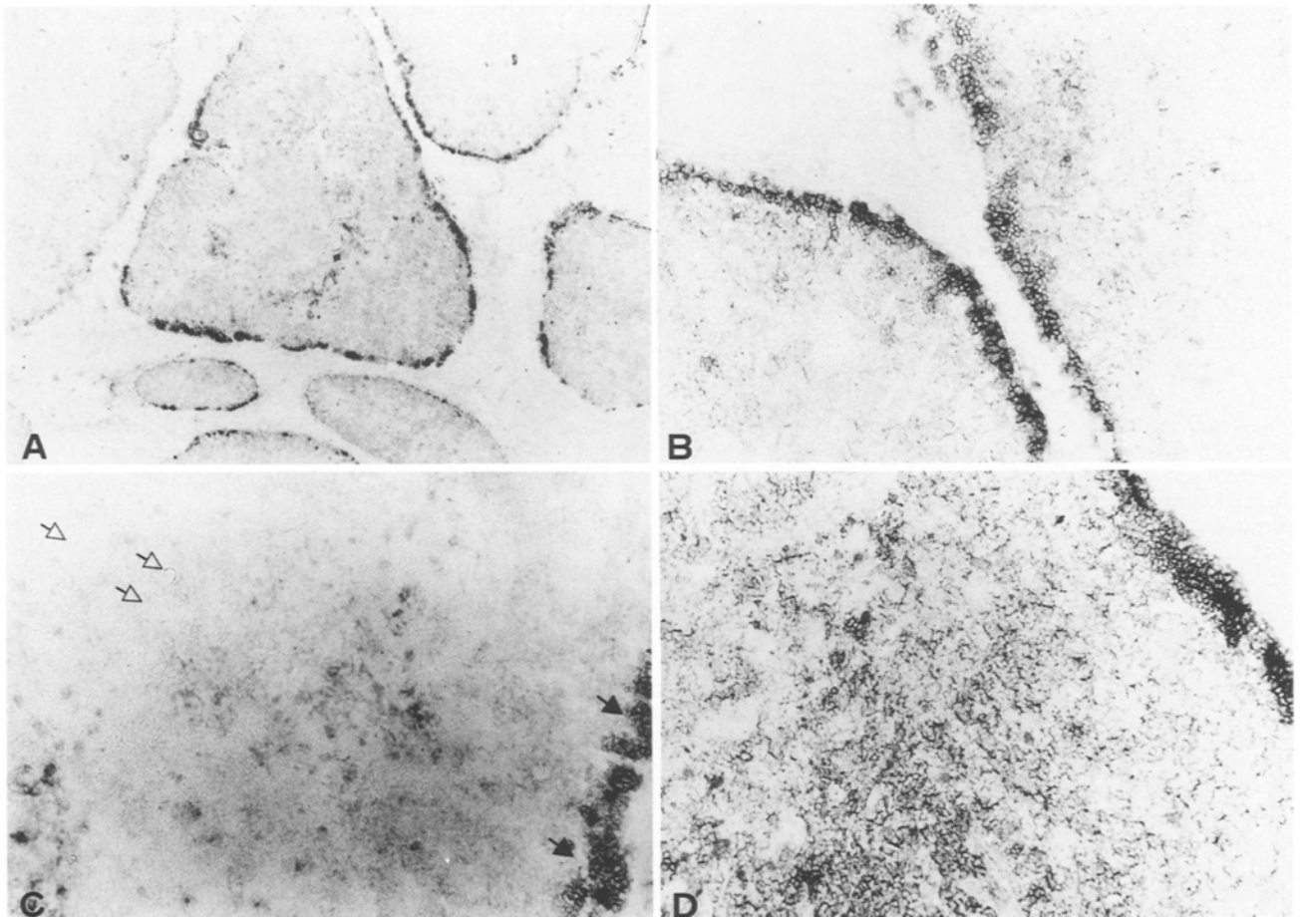

Fig. 1. Immunoperoxidase staining of a neonatal thymus with K-20 (**A** ×100, **B** ×200, **C** ×400) or anti-HLA-DR (**D**, ×400). Note the intense staining of the subcapsular cortical region by K-20 (indicated by *black arrows*). Hassall's corpuscles were not stained with K-20 (*white arrows*). In contrast, anti-HLA-DR demonstrated a strong reactivity with thymic epithelial cells in medulla and cortex

Table 3. Reactivity of K-20 with normal human tissues and cells

Organs	Reactivity	Organs	Reactivity
Liver		Thymus	
Hepatocytes	−	Subcapsular epithelial cells	+
Sinusoids	+	Other epithelial cells	−
		Hassall's corpuscles	−
Kidney		Thymocytes	−
Glomeruli	−		
Tubules	−		
Small intestine	−	Spleen[a]	−
Smooth muscle	−[a]	Thyroid gland	−
Heart muscle	−[a]		
Lung	−[a]	Adrenal gland	−

[a] Blood vessels were immunostained with K-20

Ty-82. As demonstrated in Fig. 1, K-20 was shown to react with the subcapsular cortex of four individual thymuses from newborns aged 1–5 weeks. These K-20 positive cells were also stained with anti-cytokeratin nos. 1, 5, 10, 11 and anti-HLA-DR antibodies in serial sections. The findings indicate that K-20-positive cells were thymic epithelial cells, but not thymocytes.

The subcapsular cortex is recognized as one of the sites from which bone marrow precursors first migrate (Ritter et al. 1981). This indicates that subcapsular thymic epithelial cells may be involved in the initial characterization and education of thymocytes. Antibodies to the subcapsular thymic epithelial cells have not been reported, while a number of antibodies to thymic epithelial cells have been characterized.

In a special workshop, which was held to characterize monoclonal antibodies to thymic epithelial cells of man, mouse, and rat, 22 monoclonal antibodies were classified

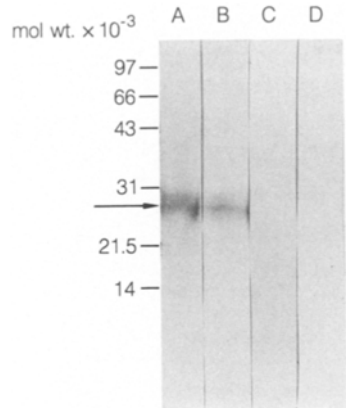

Fig. 2. Western immunoblotting of the lysates of Ty-82 (*A, C*) and thymus (*B, D*). Lanes *A* and *B* were reacted with K-20, while *C* and *D* were with control murine monoclonal antibody. A single protein band corresponding to a molecular weight of 28 kDa is demonstrated in *A* and *B*

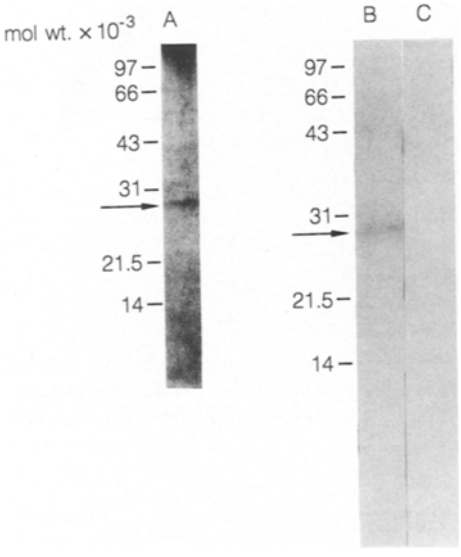

Fig. 3. Purified K-20 antigen was detected in *lane A* by silver staining under reduced conditions. A single 28 kDa protein band was detected with K-20 (*B*) but not with control antibody (*C*) under non-reduced conditions by Western immunoblotting

into five subgroups by the immunostaining pattern of the thymus; however, almost all antibodies stained the human subcapsular/perivascular and medullary thymic epithelial cells (Kampinga et al. 1989). Although De Maagd et al. (1985) reported that their monoclonal antibodies identified six distinct patterns of epithelial cell antigen expression within the human thymus, none of them (MR3, MR6, MR10, MR13, MR14, IP1, IP2, IP3, and IP4) identified the subcapsular thymic epithelial cells only. The thymosin β4, CD54 (Leu7) and p19 were restrictively expressed on the subcapsular cortex in neonatal thymus (Hirokawa et al. 1982; Haynes et al. 1983a, b; Chan et al. 1984; Kodama et al. 1986). The molecular nature of K-20 antigen and its distribution allowed us to discriminate it from the thymosin β4, CD54, and p19. Western immunoblotting and the purification procedure revealed that K-20 was directed to a protein with a molecular weight of 28 kDa. Thymosin β4 is a polypeptide, whose weight is under 5 kDa (Low et al. 1981). The molecular heterogeneity of CD57 has been reported. Anti-CD57 antibody immunoprecipitated a 110 kDa protein in T-cell lines. It also precipitated a 95 kDa and 110 kDa protein in normal mononuclear cells (Kubagawa et al. 1983). The CD57 determinant on normal thymic epithelial cells has not yet been determined; its distribution is very different from that of K-20. p19 is a structural protein of human T-cell leukaemia virus type 1 (HTLV-1) with a molecular weight (p19) obviously different from that of K-20. It appears that K-20 is a new marker of the subcapsular thymic epithelial cells.

In addition, we can discriminate K-20 antigen from the β-chain of MHC class II although both have a similar molecular weight. First, medullary and cortical thymic epithelial cells are known to express the HLA-DR, as demonstrated in Fig. 1. HLA-DQ antigen has also been reported to be expressed on thymic epithelial cells in the cortex, and slightly expressed in the medulla (Ishikura et al. 1987). In contrast to these findings, the K-20 antigen is almost exclusively expressed on the subcapsular region. Second, the splenocytes from individuals whose blood vessels demonstrated positive reactivity with K-20 were not reactive with K-20. Finally, K-20 were shown to be reactive with SP-50B but not with SP-53 derived from the same patient (Daibata et al. 1989).

K-20 was shown to be reactive with blood vessels. Blood vessels in the muscle, lung, and liver were immunostained; however, we did not observe any significant reactivity with blood vessels in the kidneys. We have not yet determined if the K-20 antigen on blood vessels is identical to that on thymus. Several monoclonal antibodies to thymic epithelial cells have been reported to be reactive with blood vessels, and it is possible that these monoclonal antibodies, including K-20, may be directed to cell adhesion molecules.

Immunofluorescent study revealed that K-20 did not react with fresh haematopoietic cells or the cell lines tested, except for SP-50B. Normal peripheral blood and bone marrow cells did not show any significant reactivity with K-20. It appears that the K-20 antigen is absent from haematopoietic cells. We cannot explain the reason why K-20 was reactive with SP-50B and not with SP-53. Type-C virus-like particles (VLPs) and Epstein-Barr (EB) virus were found in SP-50B but not in SP-53 (Kubonishi et al. 1990) and as K-20 did not immunostain other EB-virus-infected cells, this type-C VLP might be related to the expression of K-20 antigen on the haematopoietic cells. Precedents indicating the relationship of subcapsular cortical thymic epithelial cells in the newborn and type-C virus have been observed in the expression of p19 of HTLV-1. Haynes et al. (1983b) reported that anti-p19, structural protein of HTLV-1 particles, reacts with subcapsular cortical area of postnatal thymus. The hypothesis that VLPs observed in SP-50B would be involved in the K-20 antigen expression on haematopoietic cells is a fascinating one; however, it needs further extensive examination.

Unfortunately, K-20 antibody did not react with formalin-fixed and paraffin-embedded sections. We are preparing a conventional antibody to the purified K-20 antigen. This may facilitate examination of the expression of K-20 antigen on various thymomas in routinely prepared sections.

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