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A Novel Monoclonal Antibody to Thymic Medullary Epithelial Cells Raised Against a Thymoma Cell Line of the Rat

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ABSTRACT

A monoclonal antibody (mAb), RE12B2 was raised against a thymic thymoma cell line TAD1-3 and its specificity for the epithelial component of thymus assessed by immunohistochemical staining. The result revealed the mAb labeled cells in the medullary areas of the thymus. PIAS AREA study showed that the number of RE12B2 positive cells are reduced in the thymus of spontaneous thymoma rat. Western immunoblotting revealed that RE12B2 recognized a protein with a molecular weight of 35 kDa. This mAb did not exhibit reactivity with cells in peripheral lymphoid organs.

Key word: Monoclonal, Antibody, Medulla, Thymoma

INTRODUCTION

It is well established that thymic epithelial cells play an important role in T lymphocyte maturation. Induction of this maturation is mediated by production of soluble thymic hormones by the thymic stroma (1) as well as by direct cell contact and interaction between cell surface molecules expressed on stromal cells and differentiating T-cells (2). The epithelial heterogeneity was detected not only by the cell surface molecule (3), but also by the function they played during T-cell development.Recent research exhibited that the epithelium in medulla, expressing MHC class II epitope (4), was functionally distinct to that in cortex of the thymus as APC (5). Although many mAbs have been established against thymic epithelial cells, antibody restricted in medulla has been rarely reported to date. Therefore, the preparation of monoclonal antibodies to this area seems to be an important approach for the better understanding of this microenvironment.

In the present study, we established a mAb RE12B2 from a thymoma cell line to detect the medullary epithelial cell antigen. We also observed the change of this antigen in spontaneously induced thymoma.

MATERIALS AND METHODS

Animals: Inbred 6-8 week old male Wistar rats and BALB/c mice were purchased from Hokudo company (Sapporo, Japan) and were kept in the specific pathogen-free colony in our University. BUF/Mna rats were bought from the same company and brought up in the same place.

Cell lines: TAD1-3, a cell line established from a (ACI/MNS×BUF/Mna) F1 rat thymoma with a character of the medullary epithelial cells (6).

Media: All cell culture studies were performed in Sigma's RPMI 1640 medium (Sigma Chemical Co.) supplemented with L-glutamin (2×10^{-3}M) and 10% FCS (complete medium).

Production of mAb: TAD1-3 was cultured in RPMI, 37℃ for three days. Then the cells were detached by trypsin and washed three times by PBS. Two BALB/c mice were injected intraperitoneally five times with 10^7 TAD1-3 cells. Three days after the last immunization, spleen cells from the immunized mice were fused with NS-1 myeloma cells in the presence of polyethylene glycol, according to the method of Kohler and Milstein. After fusion, cells were resuspended in HAT containing complete medium at a cell density of 10^8 spleen cells and were seeded in 24-well plates. Cells were kept in HAT containing complete medium for two weeks until clones appeared.

Selection of the hybridomas: Supernatants of hybrid colonies were screened for reactivity with rat thymus by indirect immunoperoxidase staining techniques. The rats were sacrificed under ether anesthesia and thymus were obtained. Six μm frozen sections of thymus were fixed in acetone for 5 min., air-dried, washed and then incubated with the supernatants for one hour at room temperature. The immunohistochemical staining was processed according to the introduction of Histofine Kit (Nichirei Coorporation). All the sections were examined by light microscope. The hybrid cells of which supernatant stained the sections of the thymus were remained and cloned by limiting dilution. After the third cloning, the hybridomas were regarded as being established and kept for further study.

Immunochemical characterization: Isotype of the antibody was checked by mouse monoclonal antibody isotyping kit (Amersham, UK). Western immunoblotting was carried out according to the method of our laboratory. Briefly, the lysate of TAD1-3 was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore). After blocking by 5%(w/v) skim milk, the membrane was incubated with mono-clonal antibodies for one hour at room temperature. Then the membrane was incubated with peroxidase-labeled goat-anti-mouse Ig (Kirkegaard & Perry Lab. Inc., USA) for 30 min. The peroxidase was detected with

DAB-hydrogen peroxide.

Reactivity assay: The number of stained cells in every medulla was counted. The area of all medulla were detected by the computer equipped with the picture assay system (PIAS company). The criterion of the antibody reactivity was: number of positive cells/area of medulla.

RESULTS

Based on the reactivity patterns obtained in frozen sections, a hybridoma RE12B2 which produced the antibody against medulla cells was selected. RE12B2 only reacted with a few stromal cells in the medulla but failed to detect any cortical stromal or any lymphoid cells (Fig. 1a).The cells recognized were large in size and had rich cytoplasm (Fig. 1b). RE12B2 did not show any reactivity with peripheral lymphoid organs (Fig. 1c, d, Table 1), nor with several thymic epithelial cell lines examined by FACS (Table 2). Isotype analysis revealed that RE12B2 was of an IgG3 heavy chain subclass. The molecular weight of the RE12B2 antigen was determined by SDS-polyacrylamide gel electrophoresis. Lysate of TAD1-3 was employed for molecular weight analyses. A single band corresponding to a molecular weight of 35 kDa was detected under both reduced and non reduced conditions (Fig. 2). As reported, the number of every kind of cells increased progressively in thymoma (Matsuyama, 1975). Therefore, we used positive cell number/medulla area as a criterion to detect the change of reactivity of the antibody. The result of every 30 pieces of sections of normal (6w), young BUF/Mna (6w) and old BUF/Mna (1.5 years old) rats is shown in Table 3. It was found that the number of positive cells decreased in BUF/Mna rats comparing with normal rats (Fig. 3). The older the BUF/Mna rat was, the fewer the positive cells it had.

DISCUSSION

Bone marrow precursors enter the thymus and they migrate from cortical areas to medullary regions. During this process, T cells occur a series of changes in cell surface molecules. Such shifts in cell location along with differentiation suggest a series of distinct microenvironments within the thymus, each relevant to the induction of a particular stage of T lymphocyte development. There have been many monoclonal antibodies established against epithelial cells in cortical (8) and subcapsular areas (9) except antibody specific in thymic medullary area. In our study, we generated a monoclonal antibody, termed RE12B2, which reacted with epithelial cells in thymic medullary area. As demonstrated in Fig. 1, RE12B2 failed to react against any cells out of thymic medulla. The anatomical distribution pattern seemed like ER-TR5(10). But the

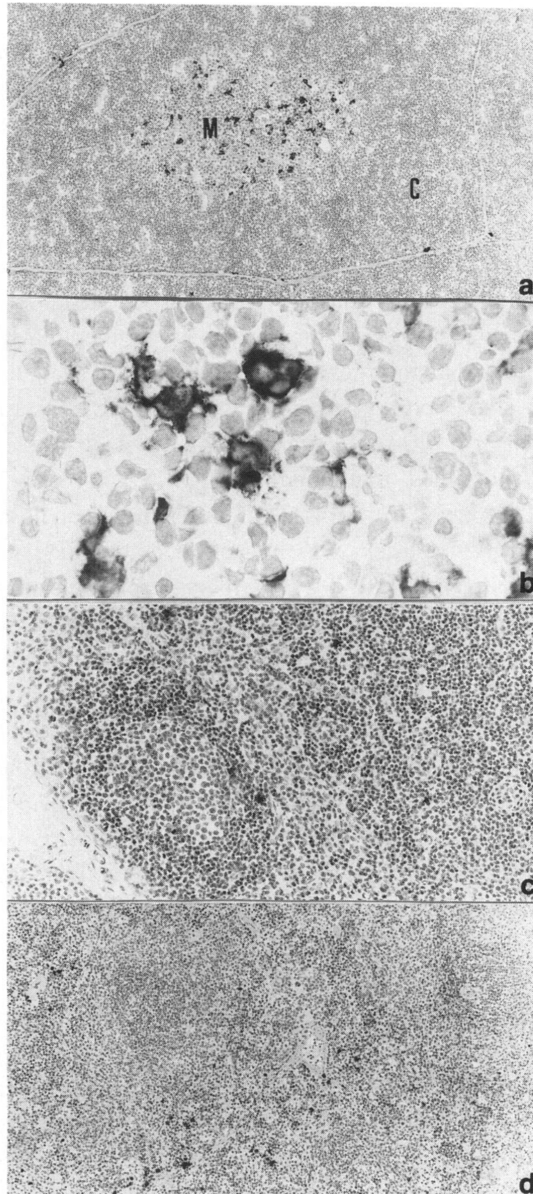

Fig. 1 Binding pattern of monoclonal antibody RE12B2 to normal rat tissues. a, A subset of medullary thymic epthelial cells displays RE12B2 positivity (×40). b, The cells recognized by RE12B2 in the medulla of the thymus (×400). c, The expression of RE12B2 in lymph node (×160). d, None of the epithelial cells was recognized by RE12B2 in spleen section except the none-specific positivity of RBC (×80).
M : medulla C : cortex

Table 1. *Extrathymic expression* of RE12B2*

Tissue	RE12B2
thymus	medulla(+)
spleen	−
lymph node	−
brain	−
lung	−
liver	−

* Analyze by immunochemical staining

Table 2. *Analysis of RE12B2 by FACS*

cell line*	RE12B2
TAD1-3	+**
TUD3	−
FTE	−
WFB	−
A3	−

* TAD1-3, TUD_3, FTE: thymic eqithelium
WFB: fetal fibroblast
A3: capillary epithelium
** cytoplasmic expression

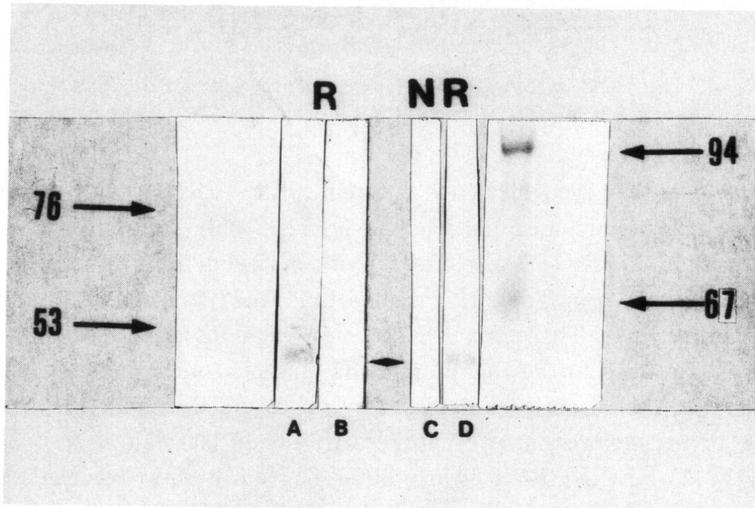

Fig. 2 Analysis of RE12B2 antigen molecular weight by western blotting under both reducing (R) and non-reducing (NR) conditions.
A, D: RE12B2 B, C: control

Table 3. *Analysis of RE12B2-Positive Cells by Picutre Assay System, PA-555*

animal strain	age (week)	number	$\frac{\text{positive cell}}{\text{medulla area(mm}^2\text{)}}$ (x+s)	P#
Wistar	6	30	243.3+59.9	
BUF/Mna	6	30	179.3+23.2	>0.05
BUF/Mna	84	30	35.0+10.7	<0.01

#: Compare with normal rats

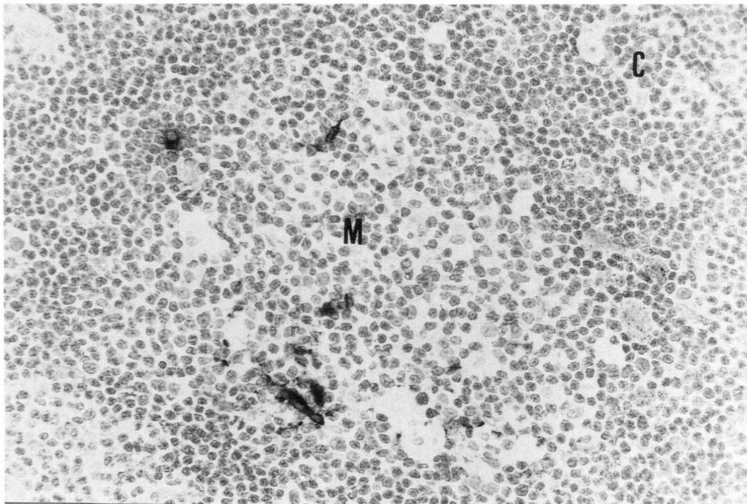

Fig. 3 The reactivity of mAb RE12B2 with the thymus of BUF/Mna rat.
M: medulla C: cortex

range of the cells they recognized are different. ER-TR5 recognizes all the epithelial cells in the thymic medulla, whereas RE12B2 only recognizes a few large cells. Furthermore, ER-TR5 also reacts with epithelial cells in various organs. But our result (shown in Table 1) revealed that RE12B2 did not react with any cells in other organs. Therefore, RE12B2 and ER-TR5 are considered to react with different proteins in the thymic medulla epithelial cells. As reported, some of the thymic epithelial antigens detected by mAbs are the subtype of keratin, in which the thymic expression of cytokeratin 18 (18,000 daltons) is similar to RE12B2 (11, 12) but RE12B2 is clearly different from keratin because of its presence in only a few cells and different molecular weight (35,000 daltons). However, the possibility still remains that RE12B2 recognizes other subtypes of keratin which we have not examined yet. In any case, above facts indicate that

RE12B2 is a specific marker of the medulla thymic epithelial cells.

This antibody also showed its expression in thymoma (Fig. 3). But the number of the cells recognized by the mAb in the thymus of BUF/Mna rat reduced statistically. Although there was also a reduction in the thymus of young BUF/Mna rat, the average number of positive cells/medulla area was more than that of the old ones. This phenomenon, therefore, suggests that the protein recognized by RE12B2 decreased corresponding reversely with the weight of the thymoma.

BUF/Mna rat develops large benign thymoma spontaneously, which is a mixture of non-neoplastic lymphocytes, macrophages and epithelial cells. And the epithelial cell line from BUF/Mna rats showed an important interaction with lymphocytes (6). It is well known that epithelial cells play the main role in T cell differentiation. Therefore, we would postulate that the RE12B2-positive epithelial cells might influence the inhibition of T cell proliferation in spontaneous thymoma. Our findings lead us to a hypothesis that the substance recognized by RE12B2 may play some role in the development of thymoma.

MAb RE12B2 established in our laboratory is a specific marker of the epithelial cells in the medulla and can be used as a tool in research of T cell defferentiation and induction of thymoma.

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REFERENCES

1. Chen W-F, Fan W. Cao LX, Zhang PX. Multiple types of cytokines constitutively produced by an established murine thymic epithelial cell line. Eur Cytokine Netw 1992, 3: 43-52.
2. Kinebuchi M, Ide T, Lupin D, Tamatani T, Miyasaka M, Matsuura A, Nagai Y, Kikuchi K, Uede T. A novel cell surface antigen in volved in thymocyte and thymic epithelial cell adhesion. J Immunol 1991, 146: 3721-3728.
3. DeMaagd RA, Mackenzie WA. The human thymus microenvironment: heterogeneity detected by monoclonal antiepithelial cell antibodies. Immunology 1985, 54: 745-750.
4. Murphy DB, Lo D, Rath S, Briuster RL, Flavell RA, Slanetz A, Janeway CA. A novel MHC class epitope expressed in thymic medulla but not cortex. Nature 1989, 338: 765-768.
5. Mizuochi T, Kasai M, Kokuho T, Kakiuchi T, Hirokawa K. Medullary but not cortical thymic epithelial cells present soluble antigens to helper T cells. J Exp Med 1992, 175: 1601-1605.
6. Masuda A, Ohtsuka K. Establishment of functional epithelial cell lines from a rat thymoma and a rat thymus. In Vitro Cell Dev 1990, 26: 713-721.

7. Lu J, Sakai Y. Establishment of Transplantable Tumor Lines and in vitro Cell Lines of Malignant Thymoma Developed in a BUF/Mna Rat. Jpn J Cancer Res1992, 83 : 618-624.
8. McFarland EJ, Scearce RM, Haynes BF. The human thymic microenvironment : Cortial thymic epithelium is an antigenically distinct region of the thymic microenvironment. J Immunol 1984; 133 : 1241-1249.
9. Takeuchi T, Kubonishi I, Ohtsuki Y, Miyoshi I. A new monoclonal antibody to human subcapsular thymic epithelial cells. Virchows Archiv A Pathol Anat 1991, 419 : 147-151.
10. Vliet EVV, Melis M, Ewijk WV. Monoclonal antibodies to stromal cell types of the mouse thymus. Eur J Immunol 1984, 14 : 524-529.
11. Laster and Itoh AJ. The human thymic microenvironment : Thymic epithelium contains specific keratins associated with early and late stages of epidermal keratinocyte maturation. Differentiation 1986, 31 : 67-77
12. Savino W, Dardenne M. Immunohistochemical studies on a human thymic epithelial cell subset defined by the anti-cytokeratin 18 monoclonal antibody. Cell Tissue Res 1988, 254 : 225-231.