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Monoclonal Antibody May Recognize Highly Evolutionary Conserved Molecule - An Immunocytochemical Study

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Abstract A monoclonal antibody, UB-13 which may react highly evolutionary conserved molecule was established. The antibody was made immunizing mice with homogenized skate brain and then cloning both with rat and skate thymocytes. The reactivity of the antibody was analyzed immunocytochemically using the indirect immunocytochemical method. The UB-13 appeared to be reactive with thymocytes of all species of vertebrates examined, such as fish, frog, turtle, chicken and rat. Cells of the nervous system, some populatons of lymphoid cells are also positive with UB-13 immunostaining. These findings strongly suggest that UB-13 is a unique monoclonal antibody which recognizes evolutionary conserved molecule.

Key Words: Monoclonal antibody, Evolutionary conserved antigen, Thymocytes, Brain, Immunocytochemistry

Introduction

The presence of evolutionary conserved molecules in the immune system and its biological significance has recently been noticed by several workers[1-4]. Since this field of science is rather new, however, we need more solid evidence and information. In addition to an advanced molecular biological technique, the monoclonal antibody method is an important strategy to analyze problem in this field. We tried to make mouse monoclonal antibodies immunizing mouse with homogenized brain of lower vertebrate, the skate, and cloning both with rat and skate thymocytes. We could raise successfully a monoclonal antibody reacting both with thymocytes and brain cells from various species of vertebrates.

Materials and Methods

1. Monoclonal antibody (Mab).

1) *Animals used to make Mab*: BALB/c strain mice were from the stock maintained at the Institute of Laboratory Animals of Yamaguchi Univeristy School of Medicine. The mice were inbred and maintained conventionally. Skate, *Raja kenojei*, an elasmobranch of cartilaginous fish, were caught in the Japan Sea.

This paper is dedicated to an emeritus Professor Kazuhiko Awaya, the former President of Yamaguchi University (May 1984 - May 1990), on the occation of his retirement.

2) *Immunization*: The skate brain was homogenized with an equal amount of physiological saline mixed with an equal volume of complete Freund's adjuvant and injected into BALB/c mice subcutaneously. Three weeks later, mice were immunized similarly. The third immunization was given intraperitoneally three weeks after the second immunization. Seven days after the third immunization, small amount of homogenized skate brain was injected into the mice intravenously. Total amount of antigen injected was 20μg per mouse. Spleen of mouse was removed for cell fusion three days after the last immunization.
3) *Cell fusion and hybridoma isolation*: NS-1 mouse myeloma cells were grown in PRMI-1640 (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% fetal calf serum (M.A.Bioproducts Co., Ltd., Maryland, U.S.A.) at 37℃ in humidified 5% CO_2/95% air. The myeloma cells (2×10^6) were fused with spleen cells (2×10^8) from the hyperimmunized mouse using polyethylene glycol 4,000 (Merck Darmstadt, F.R.G), as described elsewhere[5]. The cells were suspended at 2×10^6cells/ml in hypoxanthine/aminopterin/thymidine medium in 2 ml wells of 24-well tissue culture plates. When hybrid clones appeared, 14 days after fusion, culture media were screened for antibodies by radioimmunoassay (RIA). Cells from positive wells were cloned by limiting dilution with thymocytes (5×10^6/ml) as the feeder layer. Clones were judged stable when all wells containing a single clone were positive.
4) *Radioimmunoassay*: The cellualr radioimmunoassay (cRIA) used for the detection and analysis of Mab was as previously described[5]. Skate thymocytes and rat thymocytes were used for target cells after adjusting the cell number to 1-3 $\times 10^7$ cells/ml. The culture supernatant was incubated with an equal volume of each target cell suspension. The bound Mab was detected with ^{125}I-rabbit F(ab')$_2$ anti-mouse IgG (Cappel Laboratories Inc., U.S.A.) using Auto Well gamma system (Aloka, ARC-200).

2. Animals examined for detection of UB-13 reactive cells and preparation of tissues for immunocytochemistry.

Skate; *Raja kenojei*, frog; *Rana nigromaculata*, turtle; *Chrysemys scripta elegans*, chikens; *Gallus gallus* and rats (DA strain) were used as experimental animals to examine the localization of UB-13 reactive cells. Various lymphoid organs, brains and livers were frozed and cut 10μm sections on a cryostat, Bright OT/FAS (England) at -20℃. The sections were then fixed with cold acetone for 5 minutes. Human thymus which obtained in cardiac operation was also examined as a preliminary experiment.

3. Indirect immunocytochemical staining.

Fixed frozen sections were incubated with 10 times diluted ascitic fluid of UB-13 for 1 hour at about 20℃ and then thoroughly washed with PBS several times. Sections were further incubated with FITC labeled F(ab')$_2$ fragments of affinity purified goat anti-mouse IgG for 1 hour at 20℃. After wahsing with PBS, immuno-stained tissue sections were mounted in glycerol-PBS. Photographs were taken with fluorescence microscope (Nikon XF-EF, Japan). As control incubations, two types of mouse Mabs, UB-14 (IgG2a) and AWAI-1 (IgG1) were used instead of UB-13. UB-14 is Mab against anti-skate brain same as UB-13 but does not react with rat thymocytes. AWAI-1 is Mab against wheat α-amylase inhibitor[6].

Result

1. Production of hybridoma.

The mouse spleen which showed the highest anti-skate thymocyte antibody activity was used for cell fusion. In the stage of cloning, the hybridoma clone which has both anti-skate and anti-rat thymocyte antibody activities was chosen. Mab thus obtained was designated UB-13. Mab which have only anti-skate thymocyte antibody activity was also cloned in the same series of experiments and disignated UB-14. It will be described in detail elsewhere. Immunoglobulin subclasses of UB-13 and UB-14 were analyzed by double diffusion precipitation technique using anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies (Cappel Laboratories Inc., U.S.A.) and UB-13 was IgM and UB-14 was IgG2a.

2. Localization of UB-13 reactive cells in vertebrates.

Since UB-13 was originally established as Mab reactive both with skate and rat thymocytes, distribution of UB-13 reactive cells in various species of vertebrates was investigated to know whether UB-13 recognizes evolutionary conserved molecule

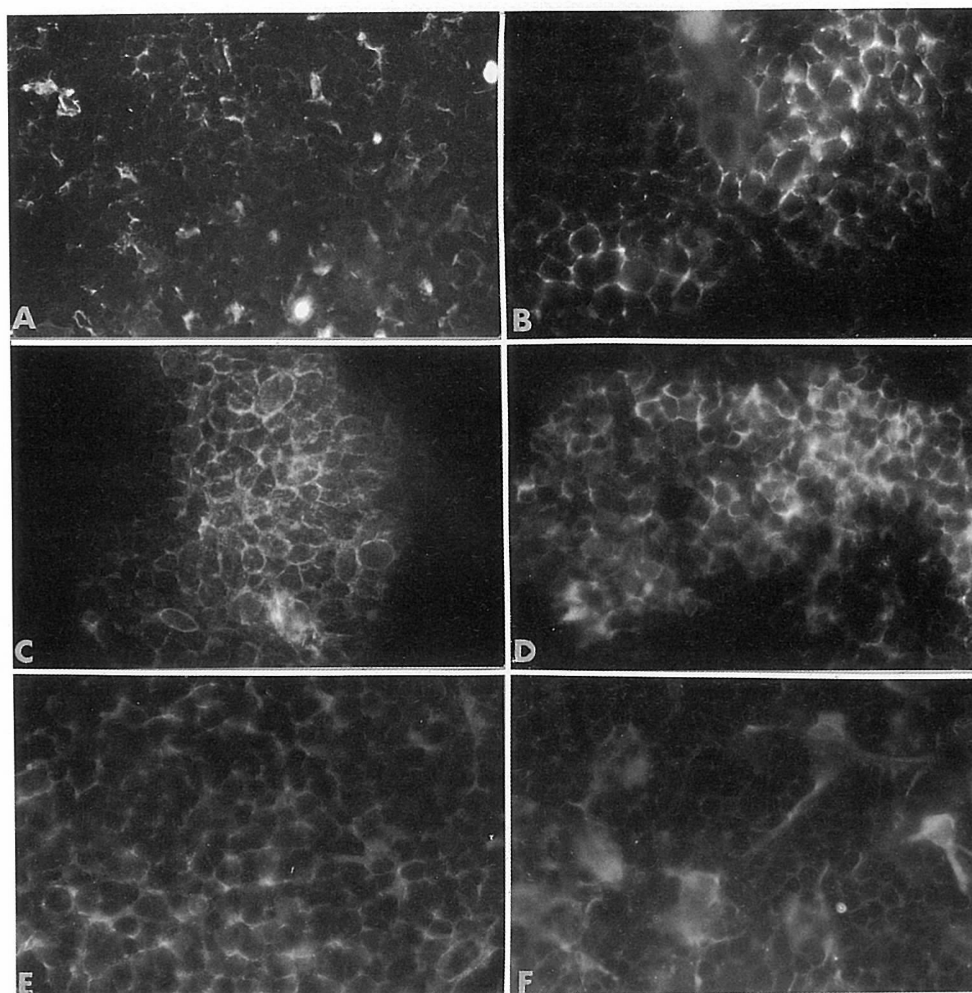

Figure 1 Indirect immunofluorescence stain of UB-13 in thymus from various classes of vertebrates.
A:Skate, B:Frog, C:Turtle, D:Chicken, E:Rat, F:Human.

throughout vertebrate phylogenetic scale. As shown in Fig. 1 and Table 1, indirect immunocytochemical analysis of UB-13 rective cells clearly show that UB-13 was reactive not only to skate and rat cells but also to the cells from the other groups of vertebrates.

1) *Thymus*: All thymus sections examined, that is of skate, frogs, turtles, chickens and rats were stained with UB-13 by the indirect immunofluorescence technique (Fig. 1). The distribution of positive staining was restricted on the cell surface, and almost no staining was observed in cytoplasm. Reticular structures were often positive for immunostaining. The pattern of immunostaining with UB-13 in human thymus tissue sections in a preliminary examination was basically similar to that of other vertebrate species, however, strong staining was often encountered in reticulum cells of human thymus (Fig. 1F).

2) *The other lymphoid tissues*: As shown in Table 1, all lymphoid organs of all vertebrates examined had a positive cell population, although the number of positive cells

Table 1. Localization of UB-13 reactive cells in various organs of vertebrates.

	UB-13	UB-14	AWAI-1
SKATE			
Thymus	++	++	−
Spleen	+	+	−
Epigonal organ	+	+	−
Leydig organ	+	+	−
Brain	++	++	−
Liver	−	−	−
FROG			
Thymus	++	−	−
Jugular body	+	−	−
Spleen	+	−	−
Brain	++	−	−
Liver	−	−	−
TURTLE			
Thymus	++	−	−
Spleen	+	−	−
Brain	++	−	−
Liver	−	−	−
CHICKEN			
Thymus	++	−	−
Bursa Fabricii	+	−	−
Spleen	+	−	−
Tonsilla cecalis	+	−	−
Brain	++	−	−
Liver	−	−	−
RAT			
Thymus	++	−	−
Spleen	+	−	−
Lymph node	+	−	−
Peyer's patch	+	−	−
Brain	++	−	−
Liver	−	−	−

UB-14 and AWAI-1 were monoclonal antibodies examined for control incubations.
++: Numerous cells were strongly positive
+: Only a small number of cells were positive
−: Negative

were different in organs and in species. Positive cells appeared to be mostly lymphoid cells morphologically and the staining was found on the cell surface.

3) *The other organs*: Cells of the brain were strongly stained with UB-13 in all species examined, however, hepatocytes were never stained with UB-13 (Table 1).

4) *Control incubation*: Mouse Mabs, UB-14 and AWAI-1, were used for control incubation instead of UB-13 in all kinds of tissue sections and no positive staining was observed except the skate cells after staining with UB-14 (Table 1).

Discussion

It is well known now that human and mouse cells have sophisticated immunorecogniton system, consisted of various molecules which belong to the Ig superfamily having similar molecular structure with immunoglobulin[4,7-10]. It was suggested that all these molecules are related in evolution

and that they diverged from a common primodial domain ancestor[1-4].

On the other hand, general occurrence of Ig-like cell surface antigens (Ig superfamily) both in brain cells and thymocytes has been reported suggesting that non-immunological recognition molecules and immunological recognition molecules may have common ancestor molecule[7-10]. Thus search for phylogenetically conserved molecules which present both in thymus and brain seems to be an important step of the research. Using skate brain as an antigen we established a monoclonal antibody, UB-13 reacting not only skate brain and thymocytes but also all thymuses and brains from all taxonomical classes of vertebrates.

Positive staining in some populations of lymphoid cells in lymphoid organs other than thymus, such as spleen, epigonal and Leydig organs in the skate, and spleen, lymph nodes and Peyer's patches in the rat, may show the localization of thymus-derived cells in such sites. It is interesting to note that in human thymus the reticular cells were stained more strongly than thymocytes, though the observation is still preliminary. Details of cytological localization of UB-13 reactive antigen in brain and thymus in various animals remain to be analyzed.

Definite finding so far obtained shows that thymocytes and brain cells have the same epitope on the cell surface which is reactive with UB-13 and the epitope is evolutionary conserved. Since biochemical analysis of the antigen(s) holding the epitope has not yet be done, it is not clear whether the presence of the same epitope represents the presence of precisely the same antigen molecule. In our preliminary study of antigen characterization, the antigen seems to be a glycoprotein. Nevertheless, it is highly possible that UB-13 is a unique monoclonal anibody recognizing evolutionary conserved cell surface antigen. The strategy of monoclonal antibody cloning with cells from phylogenetically distant species may beneficial for evolutionary study on cell surface recognition molecules.

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