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Monoclonal Antibodies against Fetal Rat Liver Cells

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Abstract The clones that produce the monoclonal antibodies to fetal rat liver cells were established by fusing spleen cells from immunized BALB/c mouse with NS-1 myeloma cells. The characteristics of the monoclonal antibodies (UB-11 and UB-12) to fetal rat liver cells were analyzed by cellular radioimmunoassay, flow cytofluorography and indirect immunoperoxidase procedures: UB-11 and UB-12 recognised preferentially the cell membrane of hemopoietic cells in the fetal liver. UB-11 might be different from UB-12, as UB-11 cross-reacted with red blood cells, while UB-12 did not. The usefulness of these two monoclonal antibodies to analyze the differentiation of the rat hemopoietic cells to lymphocytes, granulocytes or red blood cells was discussed.

Key words: monoclonal antibodies, fetal liver, cellular radioimmunoassay, flow cytofluorography, indirect immunoperoxidase procedure

Introduction

Monoclonal antibodies to adult liver cells have been established and the characteristics of each monoclonal antibody (HAM. 1, HAM .2, HAM. 3 and HAM. 4) have been reported previously[1),2)]. The amount of antigen expressions in the liver detected with these monoclonal antibodies varied following the ages of the rat. It was examined that most antigens were appeared to be expressed in low amount, during the fetal period, by cellular radioimmunoassay (Fukumoto *et al.* in preparation) or by immunohistochemical study (Tamakoshi *et al.* unpublished). The low expression of surface antigens in fetal liver may partly due to the unique cellular constituent which consists of a large number of hemopoietic cells in addition to hepatocytes. In fact, most hemopoietic cells were not reactive with HAM. 1, 2, 3 and 4. Since cell surface antigens of fetal liver cells have not been well analyzed yet, we have intended to find out the specific antigens [for either hemopoietic cells or fetal hepatocytes. The present study was designed to characterize the membrane antigens of fetal rat liver cells using monoclonal antibodies.

Materials and Methods

Animals: DA strain (RT-1 a) rats and BALB/c strain mice were from the stock maintained at the Institute of Laboratory Animals of Yamaguchi University School of Medicine. All animals were inbred and maintained conventionally.

Preparation of fetal liver cells: Fetal rats at days 15–17 of gestation were removed under Nembutal anesthesia from the uterus of pregnant DA rats. Fetal liver was obtained and placed in ice-cold phosphate-buffered saline (PBS). To prepare the cell suspension, fetal liver was teased gently using fine forceps and broken up clumps with an 1 ml syringe. Cell suspension was filtered through gauze, washed two times and resuspended in an equal volume of PBS. This cell suspension was used to immunize mice, for target cells of radioimmunoassay or for analysis by flow cytofluorography.

Immunization: The fetal liver cell suspension was mixed with equal volume of complete Freund's adjuvant and injected into female BALB/c mice subcutaneously (total cell number injected was 1–3×10^7 per mouse). Three weeks later, mice were immunized similarly. The third immunization was given intraperitoneally four weeks after the second immunization. Three days after the intravenous injection, spleen was removed for cell fusion with NS-1 mouse myeloma cells.

Cell fusion and hybridomas isolation: NS-1 mouse myeloma cells were grown in RPMI-1640 (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal calf serum (M.A. Bioproducts Co., Ltd., Maryland, U.S.A.) at 37°C in humidified 5% CO_2/95% air. The myeloma cells (2×10^7) were fused with spleen cells (2×10^8) from the hyperimmunized mouse using polyethylene glycol 4000 (Merck Darmstadt, F.R.G.), as described elsewhere [1),3),4)], in the above-mentioned medium. The cells were suspended at 2×10^6 cells/ml in hypoxanthine /aminopterin/thymidine medium[5)] in 2 ml wells of 24-well tissue culture plates. After hybrid clones had appeared, usually 10–14 days after fusion, culture media were screened for antibodies by cellular radioimmunoassay. Cultures that secreted antibodies were diluted with medium containing hypoxanthine (1×10^{-4}M) and thymidine (1.6×10^{-5} M) in microtiter plates. After growth and reassay, the cells were diluted in 2 ml wells in medium without hypoxanthine and thymidine. Cells from these wells were cloned by limiting dilution with thymocytes (5×10^6/ml) as the feeder layer[6)]. Culture medium supernatants of the wells were tested, and clones in positive wells were recloned at a density of 1 cell per well until stability had been achieved. Clones were judged stable when all wells containing a single clone were positive.

Radioimmunoassay: The cellular radioimmunoassay (cRIA) used for the detection and analysis of monoclonal antibodies was as previously described [1),7)]. Fetal liver cells and adult liver cells were used for target cells after adjusting the cell number to 1–3×10^7 cells/ml. Fetal liver cell suspensions prepared consisted of mainly hemopoietic cells and a small percentages of hepatocytes. Adult liver cells were prepared by the collagenase methods described previously[8)]. This cell suspension consisted of mainly hepatocytes and a few of non-parenchymal cells. The culture supernatant was incubated with an equal volume of each target cell suspension. The cell bound monoclonal antibodies were detected with ^{125}I-rabbit F(ab′)$_2$ anti-mouse IgG (Cappel laboratories Inc., U.S.A.) using Auto Well gamma system (Aloka, ARC-202).

The inhibition assays were performed on several kinds of tissue specimens obtained from adult DA rats. After homogenization, tissues were washed once with PBS. Diluted monoclonal antibodies were absorbed with the homogenates at various dilutions overnight at 4°C. These samples were centrifuged at 1800 rpm for 10 min. Duplicates 50 μl aliquots were then assayed by cRIA.

Flow cytofluorography: The binding profiles of the monoclonal antibodies to cell surface of fetal liver cells and adult lymph node cells were assayed with indirect immunofluorescence methods. The cells were incubated with monoclonal antibodies for 1 hour. The cell bound antibody was detected with FITC-conjugated sheep F(ab′)$_2$ anti-mouse IgG (Cappel laboratories Inc., U.S.A.) using a fluorescence activated cell sorter (FACS-Ⅲ) (Becton Dickinson Co., U.S.A.).

Indirect immunohistochemical staining : The 10 μm frozen section of tissues was incubated with 100 μl of each monoclonal antibody for 1 hour at room temperature, washed three times in cold PBS and then incubated at 4°C for 1 hour with 100 μl of horseradish peroxidase conjugated rabbit F(ab′)$_2$ anti-mouse IgG (Cappel laboratories Inc., U.S.A.). After washing with PBS, sections were incubated for 5 min with 3, 3′-diaminobenzidine HCl (Sigma Chemical Company)[2),9)].

Results

Production of hybridomas: After 3 times injections of fetal rat liver cells, the spleen of mouse which showed the highest anti-fetal rat liver cell antibody activity was used for cell fusion. The fused cells were distributed in 144 wells of six 24-well tissue culture plates. Within 10 days, 1–4 colonies were identifiable in each 33 well of the plates. Hybridomas in the antibody producing wells were cloned by limiting dilution method. Two antibody producing clones were obtained. These clones were proved stable over a period of more than 4 months in culture, or after freezing and thawing. Two monoclonal antibodies which these two clones produce were designated UB-11 and UB-12, respectively.

Characterization of UB-11 and UB-12: The binding of UB-11 and UB-12 with isolated fetal rat liver cells and adult rat liver cells was examined by cRIA. Both UB-11 and UB-12 bound very strongly to fetal rat liver cells, but not bound to adult rat liver cells (Table 1).

Table 1 Binding of monoclonal antibodies to fetal or adult liver cells.

Monoclonal antibodies tested	Binding radioactivity (cpm/assay) of monoclonal antibodies to	
	2×10^6 fetal liver cells	2×10^5 adult liver cells*
UB-11	10, 400	1, 300
UB-12	22, 300	3, 100
HAM-2	4, 700	28, 000

* Glutaraldehyde-fixed adult liver cells, which were prepared following the method described previously[1].
Background for fetal or adult liver cells were 1, 100 and 12, 800 cpm/assay, respectively.

On the other hand, HAM-2 which is another monoclonal antibody to rat hepatocytes[1], bound strongly to adult rat liver cells, but bound weakly to fetal liver cells (Table 1).

The binding of both monoclonal antibodies to lymph node cells and fetal rat liver cells were studied by flow cytofluorographical analysis with FACS-Ⅲ. Fetal rat liver cells were strongly labelled with UB-11 (Fig. 1) and UB-12, while no binding activity with lymph node cells were observed.

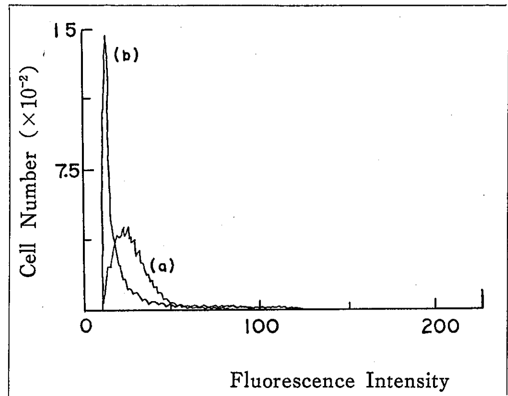

Fig. 1 FACS-Ⅲ profiles of fetal rat liver cells
Fluorescence histogram from FACS-Ⅲ analyses of freshly isolated fetal rat liver cells labelled with UB-11 (a) and control (b).

These findings were further confirmed by the binding inhibition assay using various rat tissue homogenates. UB-11 was inhibited strongly by whole fetus and fetal liver, moderately by spleen, thymus and red blood cells (RBC) and weakly by bone marrow, but not by lymph node cells and brain. UB-12 was inhibited very strongly by fetal liver, and weakly by spleen, bone marrow, whole fetus and brain, but not by RBC, thymus, lymph node cells and adult liver. The effects of the various tissue absorption for UB-12 are shown in Fig. 2. These results were summarized in Table 2.

Immunohistochemical localization of UB-11 and UB-12 antigen in fetal rat liver: The localization of UB-11 and UB-12 antigen in fetal liver was examined light-microscopically

Table 2 Characterization of monoclonal antibodies to fetal rat liver cells.

	UB-11	UB-12
Binding to		
fetal liver	++*	++
adult liver	−	−
thymus	+	−
RBC	+	−
brain	−	±
bone marrow	±	±
spleen	+	±
lymph node	−	−
Immunoglobulin subclass	IgG_1	IgG_1

*The degree of binding with each monoclonal antibody.
++; strongly positive, +; positive, ±; weakly positive, −; negative.

with immunoperoxidase staining. Fetal liver consists of hemopoietic cells and hepatocytes (Fig. 3a). Fig. 3b shows the 15 day fetal liver section stained by immunoperoxidase technique with UB-12. It is obvious that, only hemopoietic cells were stained and no staining was observed on hepatocytes. No staining was observed in the control (Fig. 3c). A similar staining pattern was observed when the section was stained with UB-11. These staining patterns were observed in the liver through the fetal and newborn periods (data not shown).

Immunoglobulin subclass: The immunoglobulin subclasses of UB-11 and UB-12 were both IgG1, when they were examined by double diffusion precipitation technique using anti-mouse IgG1, IgG2a, IgG2b, IgG3

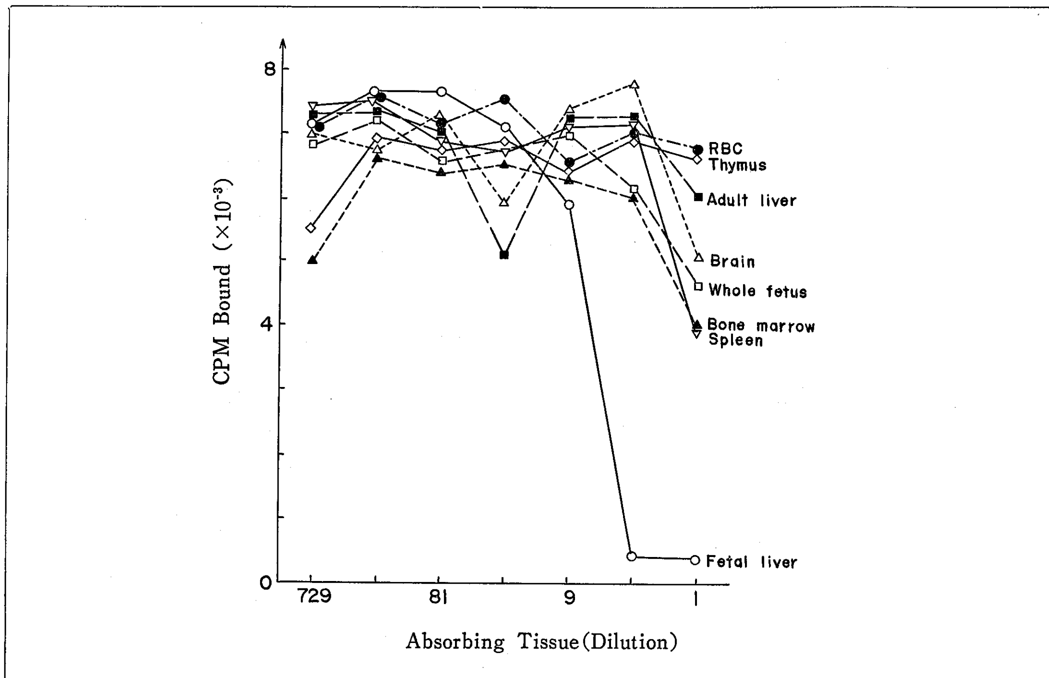

Fig. 2 Binding inhibition assay of UB-12 with several rat tissues. The degree of binding of UB-12 to fixed fetal rat liver cells was assayed after the absorption with various rat tissue or cell homogenates, is expressed by binding radioactivity (cpm/assay). Examined tissues and cells are 17 day-fetal liver, adult liver, 14 day-whole fetus, brain, spleen, thymus, bone marrow and RBC.

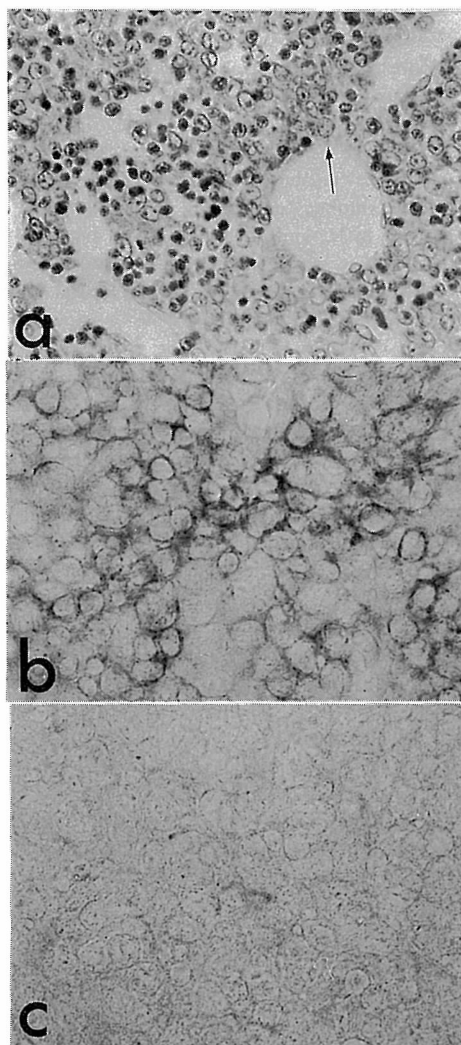

Fig. 3 Fetal rat liver section at day 15 of gestation.
a) Note a large number of hemopoietic cells and few hepatocytes (arrow). Paraffin section. Hematoxylin and eosin staining. ×360.
b) Section is stained by immunoperoxidase technique with UB-11. Note positively stained components on the surface of many hemopoietic cells, but no staining is observed on hepatocytes. Methyl-green is used for counter staining. Frozen section. ×500.
c) No staining is seen on both hemopoietic cells and hepatocytes in the control. Section is stained in the same manner as (b) except using normal mouse IgG instead of monoclonal antibody. ×500.

and IgM antibodies (Cappel laboratories Inc., U.S.A.).

Discussion

Recently, many monoclonal antibodies to rat blood cells, such as lymphocytes and granulocytes were produced, and the analyses about cell surface antigens were far developed[10)-14)]. From these results, it was suggested that rat hemopoietic stem cells carry Thy-1 antigen at an early stage of development before maturation to T and B lymphocytes. Thy-1 antigen is present on the greater part of bone marrow cells, but a small proportion of lymphocyte in the periphery. While, the cells with surface immunoglobulin are not almost detected in fetal liver or bone marrow but appear in the spleen, lymph nodes, periphery and so on. Thus, surface antigen on the lymphocytes differs from fetal and adult period in the same tissue, and appears or disappears in proportion as the hemopoietic cells emigrate from bone marrow in peripheral tissues[10)-12)]. There are quite few reports which describe the monoclonal antibody analyses for the membrane antigens of the stem cells for rat granulocytes or RBC.

The present study describes the characterization of the monoclonal antibodies, UB-11 and UB-12, which were raised against fetal rat liver cells. The characterization of these two monoclonal antibodies are summarized in Table 2. These monoclonal antibodies appeared to recognise the rat hemopoietic cells such as in the fetal liver, bone marrow and spleen. As UB-11 cross-reacted with adult rat red blood cells and thymocytes but UB-12 did not, UB-11 might be different from UB-12. It is reported that Thy-1 antigen exists in the hemopoietic cells as well as in adult thymus, bone marrow and brain but not on red blood cells or most mature lymphocytes[10)-12)]. Thus, UB-11 and UB-12 might be different from Thy-1, and these two monoclonal antibodies will be useful

for analyzing the membrane antigen of hemopoietic cells for lymphocytes, granulocytes or red blood cells, using together with Thy-1.

In these experiments, we also got a clone which produced a monoclonal antibody (HAM-5) reacting to both fetal and adult hepatocytes. This might be useful for analyzing membrane antigens of hepatocytes which exist during both fetal and adult periods.

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