

Production of Monoclonal Antibodies Against Human Colon Cancer Cell Line(SNU-C1) and Study of the Antigen.

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= Abstract = To study human colon cancer antigens, monoclonal antibodies were produced by immunizing balb/c mice with colon cancer cells(SNU-C1, originated from a Korean colon cancer patient). Among these monoclonal antibodies, one of IgM type antibody 7E02 was selected for further study. The 7E02 was proved to be effective for immunohistological staining of colorectal cancer tissues. After purification by ammonium sulfate fractionation and Sephacryl S-300 gel filtration, 7E02 was labeled by I-125. Using the radiolabeled 7E02, its dissociation constants (Kd) against SNU-C1 and SNU-C4 were measured by Scatchard plotting, and found to be 10.7 nM and 7.1 nM, respectively. When SNU-C1 was treated with 10 mM sodium periodate, 7E02 was not bound to the cell surface. However, SNU-C1 preserved the activity to bind to 7E02 after N-acetyl neuraminidase treatment. With these results, we could conclude that the 7E02 binds to carbohydrate antigen that exists on the surface of colon cancer cell and excreted mucin. The antigen was proved not to contain terminal sialic acid.

Key words: *colon cancer antigen, monoclonal antibody, carbohydrate antigen*

INTRODUCTION

Recently, immunoscintigraphy using radiolabeled cancer-specific monoclonal antibody has been

used extensively all over the world(Kim *et al.*, 1980; Chung *et al.*, 1993). For the immunoscintigraphy, development of monoclonal antibody against tumor antigen is one of the most critical step.

It has been known for a long time that cancer patients have special material increased in their body fluid. These materials are called tumor marker. Some tumor markers are common materials those can be found in normal person, such as hormones, enzymes, serum proteins and protein hydrolysates(Deland and Goldenberg 1985). However, others are very uncommon in normal adult's

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body. They are found mainly in tumor tissues or fetal tissues. These materials are called oncofetal antigens. Study of these oncofetal antigens became very active after the detection of alfa-fetoprotein (AFP; Abelev *et al.*, 1963) and carcinoembryonic antigen (CEA; Gold and Freedman 1965). Distribution of colon cancer antigen CEA in tumor tissue has been studied extensively using quantitative autoradiography (Chung *et al.*, 1994).

Many kind of oncofetal antigens exist on the surface of cancer cells. Monoclonal antibodies against these antigens can be produced by immunizing animals with cancer cells. One of the most famous colon cancer antigen which was proved by monoclonal antibody method is CA19-9. Monoclonal antibody against CA19-9 was produced by immunizing balb/c mice with a colon cancer cell line SW1116 (Koprowski *et al.*, 1978). Its molecular structure was proved to be sialylated Lewis a antigen by Magnani *et al.* (1981, 1982). Its presence on the normal pancreatic epithelia and normal gastric membrane was proved by immunohistological staining method (Atkinson *et al.*, 1982). Mucin type CA19-9 was detected in the sera of gastrointestinal cancer patients (Magnani *et al.*, 1983).

In this experiment, we tried to produce monoclonal antibodies against colon cancer cell line SNU-C1 which was derived from Korean male patient, and studied some properties of produced monoclonal antibody and its antigen.

MATERIALS AND METHODS

1. Cell Lines

Human colon cancer cell lines, SNU-C1 and SNU-C4 were used for immunization of balb/c mice and cell binding assay of monoclonal antibody. Both of these cell lines were originated from Korean male colon cancer patients (Park *et al.*, 1984, 1985).

Mouse myeloma cell line, P3/U1 was used for making hybridoma cell line. This cell line was originated from balb/c myeloma and proved to be non-secretor of serum proteins.

All the cells used in this experiment were incubated in RPMI1640 medium containing 5 % fetal

bovine serum and maintained in 37°C incubator with 5 % CO₂ enrichment.

2. Reagents and Instruments

The RPMI1640 cell culture media and fetal bovine sera were purchased from Gibco company. Anti-mouse antibody goat antibody was from Sigma company. Sephadex G-50 and Sephacryl S-300 were purchased from Pharmacia company. I-125-Nal was obtained from DuPont NEN company. Other reagents for buffer solutions were obtained from Sigma or Wako company. Gamma scintillation counter (Multi-prias) was from Packard company. CO₂ incubator (KMC-8409C) was manufactured by Korea Manhattan company.

3. Preparation of Hybridoma

Each balb/c mouse was injected by 10⁷ cultured SNU-C1 cells four sites on the back subcutaneously. Booster injections were given 15 days and 20 days later by same number of cells intraperitoneally. Four days after the second booster dose, spleen cells were obtained from the immunized mouse and fused with P3/U1 myeloma cells (Kohler and Milstein 1975). The hybridoma were selected by hypoxanthine, aminopterin and thymidine enriched media. Monoclonal antibodies secreted in hybridoma supernatants were screened by cultured SNU-C1 cells and I-125 labeled second antibody (anti-mouse antibody goat antibody). Eleven monoclonal antibodies those can bind to surface of SNU-C1 were selected by this method. These hybridoma were cloned by limited-dilution method using normal balb/c mouse spleen cells for conditioning medium. The cloned hybridoma were harvested and stored in liquid nitrogen after frozen in a medium composed of fetal bovine serum : RPMI1640 medium : dimethylsulfoxide = 50 : 40 : 10.

4. Determination of Sub-Isotypes of Monoclonal Antibodies

Anti-mouse immunoglobulins (IgG, IgA, and IgM) goat antibody (Sigma Co, 100µl, 0.05 mg/ml in 0.05 M sodium carbonate buffer, pH 9.5) was coated to 96-well ELISA plate by incubating overnight at 4°C. Fifty µl of phosphate buffered saline

(PBS) containing 0.5 % Tween 20 and 50 μ l of cloned hybridoma supernatants were added to the antibody-coated wells and incubated for 1 hour at room temperature. After washing with PBS containing 0.5 % Tween 20 three times, 2 drops of each biotin-conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM solution (Amersham Co) were added and incubated for 1 hour at room temperature. After washing with PBS containing 0.5 % Tween 20 three times, 100 μ l of avidin-peroxidase conjugate solution (Sigma Co, diluted 1:500 in PBS) was added to each well and incubated for 1 hour at room temperature. After washing with PBS containing 0.5% Tween 20 three times, 50 μ l of o-phenylenediamine solution containing 0.1 % urea-hydrogen peroxide was added and incubated for 10 to 20 minutes in the dark. Development of orange color was stopped by adding 50 μ l of 1 N hydrochloric acid.

5. Immunostaining of Colon Cancer Tissues

Thickness of 5 μ m sections on slide glasses for microscopic investigation were prepared from formalin-fixed paraffin-embedded colon cancer tissues. The sections were hydrated by dipping in xylene, absolute ethanol, 90% ethanol, 70% ethanol, 50% ethanol, and PBS, sequentially. Possible peroxidase activity existing in the tissue preparation was destroyed by treatment in 0.3% hydrogen peroxide in ethanol, and washed by PBS again. Hybridoma supernatants were added on the top of the prepared tissue-sections, and incubated overnight at 4°C in humid chamber. After washing with PBS 3 times, biotinylated anti-mouse immunoglobulins goat antibody (diluted 1:500 in PBS) was added and incubated for 1 hour at room temperature. After washing with PBS 3 times, avidin-peroxidase (diluted 1:200 in PBS) was added and incubated for 1 hour at room temperature. After washing with PBS 3 times, AEC solution containing 0.1% urea-hydrogen peroxide was added and incubated for 15-25 minutes at room temperature. Excessive development of color was stopped by washing in tap water. The immunostained sample was stained again with hematoxylin.

6. Purification and I-125 Labeling of Monoclonal Antibody

Cultured hybridoma (10⁷ cells) were injected into the peritonia of pristane-primed balb/c mice, and ascitic fluids were obtained from the balb/c mice about 1 week later. Monoclonal antibody was purified from the pooled ascitic fluid by precipitation with 50% saturation of ammonium sulfate twice and successive gel-filtration with Sephacryl S-300 column chromatography (1.5 cm \times 100 cm). The purity of monoclonal antibody was confirmed by SDS-PAGE. To study binding constants, the purified monoclonal antibody was radiolabeled by I-125 (Hunter and Greenwood 1962).

7. Scatchard Plotting of Monoclonal Antibody

Cultured colon cancer cell line SNU-C1 (1.8 \times 10⁵ cells) or SNU-C4 (2.0 \times 10⁵ cells) were incubated with serially diluted I-125 labeled monoclonal antibody for 1 hour at room temperature. The cell bound radioactivity was measured by gamma scintillation counter after washing the incubated cells with PBS. Affinity constants were determined by Scatchard plotting.

8. Treatment of SNU-C1 with Neuraminidase and Periodate

To study the chemical properties of the cell surface antigen, SNU-C1 was treated with neuraminidase (1 Unit, 1 hour at 37°C) or periodate (20 mM, 1 hour at 37°C) and tested for the binding of monoclonal antibody (Coligan and Todd 1975).

RESULTS

Immunization of balb/c mice was performed by subcutaneous injection of SNU-C1 cells and subsequent booster injection into peritoneum. Harvesting SNU-C1 cells from cultured media was simple, because the cells were not bound to the surface of culture bottle. However, to harvest SNU-C4 that grows bound to the surface of culture bottle, trypsinization was necessary. Spleen cells of the immunized mice and a mouse myeloma (P3U1) were fused by PEG method. The ratio between

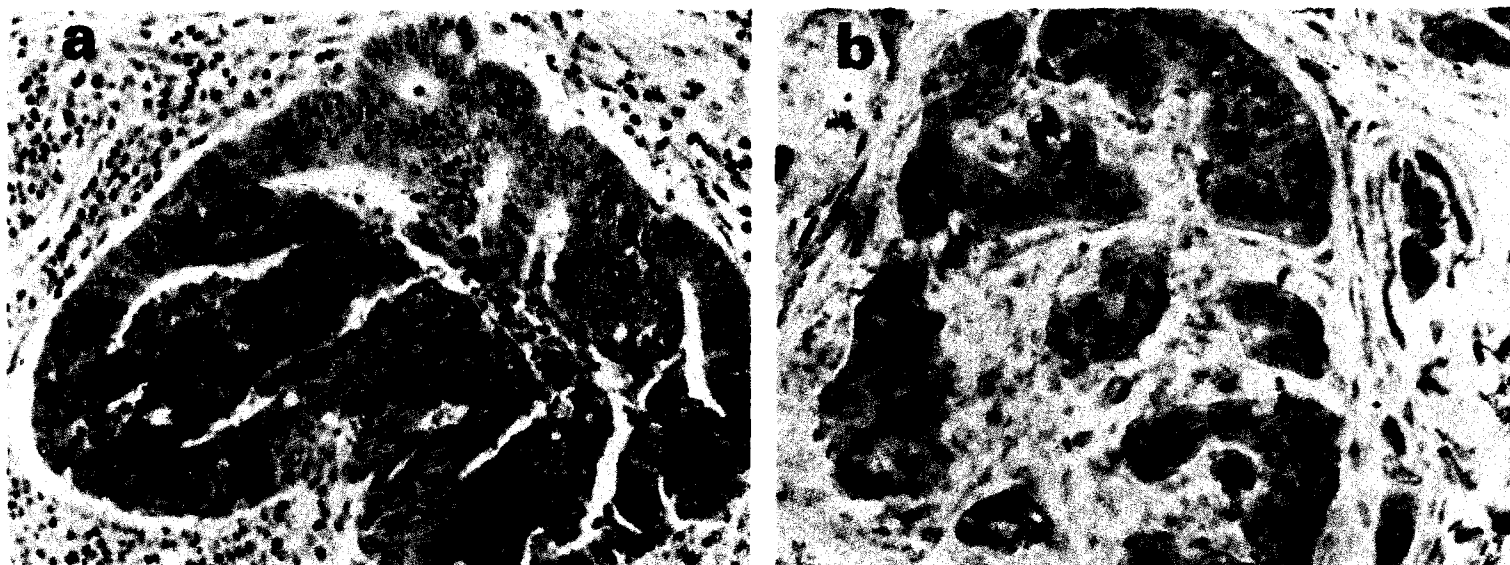


Fig. 1. Microscopic views of human colon cancer tissues immunohistologically stained by 7E02. Each formalin-fixed tissue was treated in tap water overnight to eliminate formalin. Blocks were made with these tissues by embedding in paraffin, sectioned to $5\mu\text{m}$ sections by microtome, and the sections were loaded onto slide glasses. The paraffin was eliminated by dipping in xylene 3 times for 5 minutes each. The tissue-bound slide glasses were treated by absolute ethanol, ethanol containing 0.3% hydrogen peroxide (destroy endogeneous peroxidase), 90% ethanol, 70% ethanol, 50% ethanol, and PBS sequentially for 10 minutes each. The 7E02 was applied to the treated sample and incubated for 1 hr at room temperature in humid chamber, and the slide glasses were washed by PBS 3 times for 10 minutes each. Biotin-labeled anti-mouse goat antibody and avidin-peroxidase conjugate were mounted as the same way sequentially. AEC solution containing 0.1% urea-hydrogen peroxide was applied to the treated sample and incubated for 15 to 25 minutes, and the slide glasses were washed by PBS twice for 10 minutes each. After counter-staining with hematoxylin, photographs were taken under a microscope. Each formalin-fixed tissue is: a, well differentiated adenocarcinoma; b, mucinous carcinoma; c, normal colon tissue.

spleen cell and myeloma to achieve best hybridization yield was found to be about 8:1.

The fused cells were dispensed into 192 wells and 151 wells showed growth of hybridoma. Among the 151 hybridoma, 11 of them showed SNU-C1 binding activity. Only 3 of them showed applicability for immunostaining. Among the 3 monoclonal antibodies, one (7E02) was selected and studied further. The 7E02 was proved to be

IgM by the method described above.

Immunohistogram shows that 7E02 binds to well differentiated colon cancer tissue heterogeneously (Fig. 1a). It binds mucin as well as cell surface in mucinous carcinoma (Fig. 1b), however, does not bind to normal colon tissue (Fig. 1c). Anti-CEA antibody also binds to both mucine and cell surface.

The dissociation constant (K_d) of 7E02 to SNU-

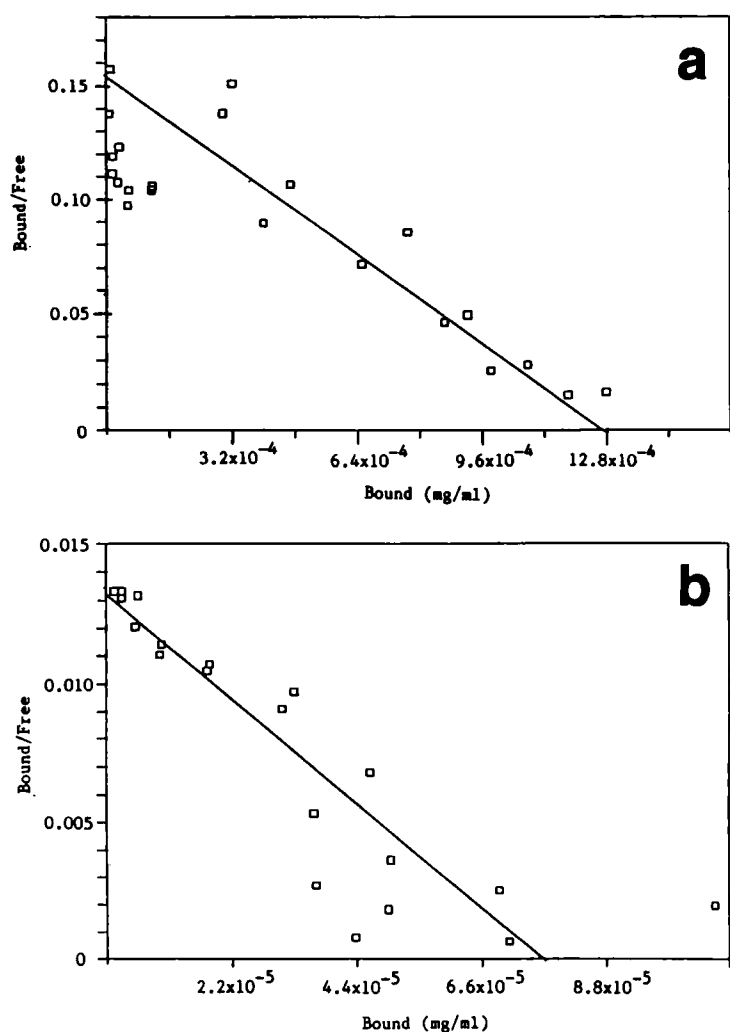


Fig. 2. Scatchard plotting of 7E02 binding to: a, SNU-C1 cells; b, SNU-C4 cells. Ratio of bound to free I-125 labeled 7E02 plotted against amount of bound I-125 labeled 7E02 expressed as mg/ml of 7E02. The K_d value and the maximum binding capacity were determined by slope and x-intercept, respectively.

C1 and SNU-C4 were 10.7 nM and 7.1 nM, respectively (Fig. 2). These results show moderate affinity of monoclonal antibody to the colon cancer cells. Number of epitopes on the surface of SNU-C1 and SNU-C4 were calculated as 3.3×10^6 /cell and 1.3×10^5 /cell, respectively (Fig. 2).

After treatment of SNU-C1 with N-acetyl neuraminidase at 37°C for 1 hour, no alteration in the binding activity of 7E02 to SNU-C1 was observed (Fig. 3). However, treatment with sodium periodate destroyed the binding activity of 7E02 to

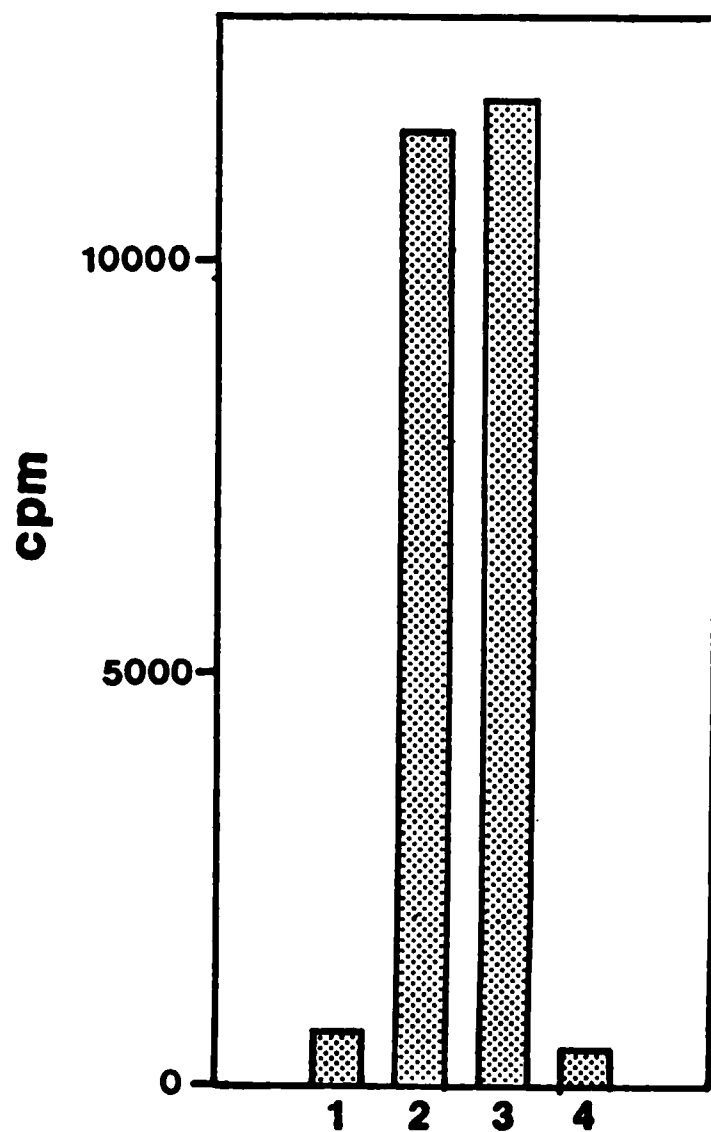


Fig. 3. Binding of 7E02 to SNU-C1 cells treated with neuraminidase or sodium periodate. SNU-C1 cells (3×10^5) were treated with 1 U neuraminidase or 10 mM sodium periodate for 1 hour at 37°C in the dark, washed with 2 ml of PBS, incubated with 400 μ l of I-125 labeled 7E02 (11,600 cpm) for 1 hr at room temperature, centrifuged at 3,000 rpm, and counted the radioactivities of the precipitated cells with scintillation counter. Non-specifically bound radioactivity: 1, was measured by counting non-treated cells incubated with 1 mg/400 μ l of non-labeled 7E02 and I-125 labeled 7E02 (11,600 cpm). Method to determine total bound activity: 2, was the same as that of non-specifically bound activity except the absence of non-labeled 7E02. Cells are treated by: 3, neuraminidase; 4, sodium periodate.

SNU-C1. These results show that the antigen is composed of carbohydrate that does not contain terminal N-acetyl neuraminic acid.

DISCUSSION

There are normal antigens as well as cancer antigens on the surface of cancer cells. Hybridoma technology is one of the best method to identify cancer antigens existing on the surface of cancer cells. A lot of hybridoma which produce monoclonal antibodies against normal antigens or non-specific antigens can be eliminated by selecting adequate screening method. We used colon cancer cells which was used for immunizing the balb/c mice and colon cancer tissues to screen the specificity of produced monoclonal antibodies in this experiment.

Glycoprotein or glycolipid is the most common chemical nature of cancer antigens. Two most famous glycoprotein cancer antigens are alfa-fetoprotein and CEA. Alfa-fetoprotein exists high concentration in the sera of fetus and hepatoma patients. CEA is found in human gastrointestinal adenocarcinoma or fetal gastrointestinal tissue with molecular weight 180,000 dalton (Gold and Freedman 1965). Bast *et al.*, produced monoclonal antibody OC125 and OC133 against ovarian cancer (1981). The OC125 and OC133 were proved to be glycoprotein with molecular weight > 200,000 and 80,000 dalton, respectively (Masuho *et al.*, 1984). A melanoma antigen p97 which has sequence homology with transferrin or lactotransferrin was proved to be a glycoprotein with molecular weight 97,000 dalton (Brown *et al.*, 1982). According to their high molecular weight and complexity, glycoprotein antigens are more difficult to study the chemical nature than glycolipid antigens.

On the other hand, glycolipids antigens are easier to analyze and studied more systematically than glycoprotein antigens. Mainly, three kinds of changes were found in glycolipid when normal cells transformed to cancer cells: i) accumulation of rare glycolipids due to blockade of biosynthesis of certain glycolipid, ii) synthesis of new glycolipid according to the activation of normally inac-

tive glycosyl transferase, and iii) revealed glycolipid, which has been hidden by other antigens in normal cells, due to physicochemical changes in cell membrane (Hakomori 1984, 1985).

If animals are immunized by cancer cells, then the resulting antibodies will be produced against carbohydrate side chain of glycoproteins or glycolipids. According to our results, epitope of 7E02 antigen is proved to be carbohydrate existing both on the cell surface of colon cancer and mucus of mucinous carcinoma. However, it is not proved whether the antigen is glycoprotein or glycolipid.

In conclusion, we could successfully produce monoclonal antibody against colon cancer cell (SNU-C4), and the antibody specifically binds to carbohydrate epitope which exists colon cancer tissues and mucin.

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