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Identification of a Malignant Cell Associated Antigen Recognized by a Human Monoclonal Antibody*

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Abstract—*A human × human hybridoma, CLNH11, derived from a lymphocyte of a patient with cervical carcinoma, produces a human monoclonal antibody of* γ_1 *and* κ *isotypes (CLN-IgG). Immunoperoxidase staining showed that CLN-IgG reacted with frozen tissue sections of human malignant tumors (cervical carcinoma, gall bladder carcinoma, glioblastoma), but not with their normal counterparts. Enzyme-linked immunosorbant assay also demonstrated that CLN-IgG reacted with various human tumor cell lines, but not with non-tumorigenic cells such as some fibroblasts, peripheral blood lymphocytes and red blood cells. Indirect and direct immunofluorescence staining indicated that the tumor antigens recognized by CLN-IgG were located in restricted areas close to the cell surface and exposed on the outer surface of the cell membrane. A protein antigen of* M_r 226,000 *was purified to homogeneity by affinity chromatography with CLN-IgG from the plasma membrane fraction of A549 lung tumor cell line. The antigen consisted of* α (M_r 60,000) *and* β *subunit* (M_r 53,000) *which were linked by disulfide bond(s) (TA60K/53K). The TA60K/53k antigens were expressed commonly in other tumor cell lines originated from histologically different tissues.*

INTRODUCTION

TUMOR IMMUNOLOGY is based on a concept that malignant tumor cells express unique cell surface antigens which are not found in normal cells, and that immune surveillance recognizes such antigens and in some cases eliminates the cells carrying them.

In humans, serological studies have provided evidence for the presence of tumor specific or associated antigens [1, 2], but their biochemical characters were unclear owing to the heterogeneity of antibodies in the serum.

Since hybridoma technology [3] was established in the past decade, a number of murine monoclonal antibodies (MoAbs) to human tumor cells have been made and many antigens have been identified [4–6]. Most of these MoAbs, however, were derived from spleen cells of mice or rats immunized with whole human tumor cells or cell extracts; thus, the results may not directly reflect the intrinsic immunogenicity of human tumor cell antigens in a tumor-bearing host.

Therefore, it is of worth investigating human tumor antigens by human MoAb produced from immortalized human B lymphocytes which were immunized in an autologous system. Recently, several human MoAbs against human tumor cells have been produced by using cancer patients' lymphocytes [7–13].

Hagiwara and Sato [10] reported the production of human MoAb CLN-IgG which reacted with the autologous cervical carcinoma. However, little was reported about the molecular nature of the tumor associated antigen in the previous paper. In this report, we studied the cellular localization and identification of the antigen recognized by CLN-IgG.

MATERIALS AND METHODS

Hybridoma production

The methods for generating human × human hybridoma (CLNH11) were reported previously [10]. Briefly, peripheral blood and lymph node lymphocytes of a patient with cervical carcinoma were fused with human B-lymphoblastoid cell line UC729-6 [9] by use of polyethylene glycol 1500. The one clone CLNH11 among the resulting six clones produced human monoclonal IgG_1 (CLN-

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IgG) having κ light chain. CLNH11 was recloned ten times by the limiting dilution method.

Human cell lines and blood cells

HeLa229, CaSki, ME-180 (cervical carcinomas), A549 (lung carcinoma), G361 (melanoma), PC-3 (prostate carcinoma), U-373MG (glioblastoma), WI38, Detroit 551, MRC-5, MRC-9 (fibroblasts) and IM-9 (B-lymphoblastoid cell, IgG producer) were purchased from American Type Culture Collection (ATCC; Rockville, MD, U.S.A.). AGS and MKN45 (stomach carcinomas) were from Dr. M. Glassy, University of California San Diego, Cancer Center. Peripheral blood lymphocytes were isolated by use of Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) and A^+, B^+, AB^+ and O^+ type human red blood cells were isolated from normal adult individuals.

Culture of hybridoma and human established cell lines

CLNH11 and IM-9 were maintained in serum-free medium (Hybrity-1; Sanko Chemical Co., Tokyo, Japan) [14] or RDF medium (2:1:1 mixture of RPMI 1640, Dulbecco's modified Eagle's and Ham's F-12 medium) containing 5% fetus bovine serum (FBS). Other human established cell lines were cultured in DF medium (1:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 medium) containing 10% FBS. RDF and DF media were supplemented with streptomycin (50 μg/ml), penicillin (50 U/ml) and ampicillin (50 μg/ml).

Purification of human monoclonal antibodies

Monoclonal antibody in the serum-free culture medium of CLNH11 or IM-9 was precipitated with ammonium sulfate at 70% saturation. The precipitate was then collected at 10,000 ***g*** for 20 min, resuspended in and dialyzed against 10 mM phosphate buffered saline (PBS; pH 7.2). Dialyzed solution was adsorbed to protein A coupled to Sepharose 4B, followed by washing thoroughly with PBS. The adsorbed material was eluted with 0.2 M glycine–HCl (pH 2.8). The eluted fraction was passed through Sephadex G-25 equilibrated with PBS, lyophilized and stored at −20°C before use. The purity of the antibodies were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [15] and high performance liquid chromatography. Protein was measured by the method of Lowry *et al.* [16].

Enzyme-linked immunosorbent assay (ELISA)

When adherent cells were used as the target, cells were treated with 0.25% trypsin and 0.02% EDTA for 30 min at 37°C. The cells detached from the dish were collected, washed with DF medium and suspended in DF medium containing 10% FBS. Fifty thousand cells were put into each well of 96-well microtiter plates (Corning Glass Works, Corning, NY, U.S.A.) and incubated overnight at 37°C in a humid, 5% CO_2 atmosphere. When nonadherent cells were used as target, cells were washed with and suspended in DF medium. Fifty thousand cells were put into each well of 96-well microtiter plates and incubated overnight at 37°C in a humid, 5% CO_2 atmosphere.

After the plate was centrifuged at 1500 rpm for 10 min, the cells were fixed with cold 2% paraformaldehyde in PBS for 30 min at 4°C and washed three times with PBS containing 0.3% gelatin and 0.02% thimerosal (gelatin buffer). The fixed cells were covered with 5% (w/v) bovine serum albumin (BSA, fraction V; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS containing 0.02% thimerosal (BSA buffer), washed with gelatin buffer and incubated with 50 μl of CLN-IgG or IM-9 IgG at a concentration of 4 μg/ml for 1 h at 37°C. After six washes with gelatin buffer, 50 μl of peroxidase conjugated anti-human IgG (γ chain specific; TAGO Inc., Burlingame, CA, U.S.A.) at 1:2000 dilution was added and incubated for 30 min at 37°C. Then, 50 μl of peroxidase substrate (*o*-phenylene diamine, 0.4 mg/ml; H_2O_2, 0.006%) was added after washing.The reaction was stopped by adding 5 N H_2SO_4 (50 μl) after a 20 min incubation in the dark at 37°C. And the absorbance at 492 nm was read with an ELISA reader (Corona Electric, Katsuta, Japan).

Indirect immunofluorescence staining

Cells were grown on glass coverslips in DF medium containing 10% FBS. Before confluence, the cells were washed with PBS and fixed with a 3.7% solution of formaldehyde in PBS for 25 min at room temperature. After washing with PBS, the cells were incubated with 5% BSA buffer for 30 min at room temperature. Then cells were incubated with 10 μg/ml of purified CLN-IgG for 1 h at 4°C. After washing with PBS, the cells were stained with FITC-conjugated goat anti-human IgG (1:10; Cappel Laboratories, Cochranville, PA, U.S.A.) for 30 min at 4°C. Stained cells were photographed under a fluorescence microscope (Nikon, Tokyo, Japan).

Direct immunofluorescence staining

Cells were grown in microglass chambers (Lab-Tek; Miles, Naperville, IL, U.S.A.) in DF medium containing 10% FBS. Subconfluent cultures were fixed with 2% paraformaldehyde for 1 h at 4°C. The fixed cells were washed with PBS and incubated with 5% BSA buffer for 1 h at 4°C. The covering solution was discarded and the cells were treated with 0.02% trypsin solution for 20 min at 37°C. Thereafter, the cells were washed with a DF medium

containing 10% FBS and allowed to stand for 30 min in the same medium. The medium was discarded and the cells were stained with CLN-IgG-coupled fluorescent beads (150 μl) for 1 h. FBS (200 μl) was added and allowed to stand for 1 h. The serum was aspirated and the cells were washed three times with PBS. The stained cells were viewed under a fluorescence microscope (Nikon).

CLN-IgG-coupled fluorescent beads were prepared as follows: 100 μl of Covasphare-MX suspension (0.7 μm; Covalent Technology Corporation, Redwood City, CA, U.S.A.) was washed with 0.2% EDTA and then resuspended in 100 μl of distilled water. Ten microliters of CLN-IgG (1.2 mg/ml in PBS) was added to the suspension and incubated for 2 h with agitation. The antibody-coated beads were washed three times with 1% BSA buffer, suspended in 100 μl of 1% BSA buffer containing 0.02% NaN_3 and stored at 4°C until use.

Immunoperoxidase staining

CLN-IgG was biotinylated by the method of Heggeness and Ash [17]. Frozen tissue sections (8 μm) were fixed in acetone for 10 min at room temperature, then washed with 10 mM PBS for 20 min. Sections were incubated with normal human serum for 20 min, blotted to remove excess serum and incubated with biotinylated CLN-IgG (10 μg) for 45 min at 30°C in a humid atmosphere to prevent drying. Sections were washed for 10 min in PBS, incubated with avidin-biotinylated peroxidase complex (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 45 min at 30°C, washed in PBS and incubated in peroxidase substrate (diaminobenzidine, 0.26 mg/ml; H_2O_2, 0.05%) for 5 min. Sections were then washed with water, counterstained with hematoxylin and examined microscopically.

Purification of tumor cell antigens

Plasma membrane was isolated by the method of Thom *et al.* [18] from tumor cells which were grown in nude mice or cultured *in vitro*.

Plasma membrane of the tumor cell was mixed with 5% (w/v) Brij 96/99 (1:2 mixture of Brij 96 and Brij 99) (Sigma, St. Louis, MO, U.S.A.) in PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and incubated overnight at 4°C with constant stirring. The solution was then centrifuged at 80,000 ***g*** for 75 min at 4°C. The supernatant was applied to CLN-IgG-coupled Sepharose 4B column. After washing the column with 5% Brij 96/99 containing 1 mM PMSF, the absorbed material was eluted with 3.5 M $MgCl_2$ in 5% Brij 96/99 containing 1 mM PMSF at 4°C. Molecular weight of the antigen was determined by SDS–PAGE followed by silver staining [19].

RESULTS

CLN-IgG antibody

Human × human hybridoma, CLNH11, produces IgG (γ_1,κ) in the serum-free medium at a rate of 6.2μg/ml/10^6 cells/24 h. The secreted IgG was purified by affinity chromatography using protein A-Sepharose. The purified IgG (CLN-IgG) was analyzed on SDS-PAGE under reducing conditions, together with an irrelevant human monoclonal IgG (γ_1,κ) which was produced by B-lymphoblastoid cell line IM-9 and purified by the same method as that used for CLN-IgG purification (Fig. 1). Only two bands corresponding to heavy (M_r 55,000) and light chain (M_r 25,000) were observed from each IgG.

Reactivity of CLN-IgG with human tissue sections

CLN-IgG was biotinylated and used for immunoperoxidase staining of frozen tissue sections of human malignant tumors and their normal counterparts. As shown in Fig. 2, biotinylated CLN-IgG bound to cervical carcinoma, gall bladder carcinoma and glioblastoma. In contrast, it did not react with normal cervix, gall bladder and brain. This suggested that CLN-IgG could recognize the epitope on the molecule closely associated with malignant tumor.

Binding activity of CLN-IgG to tumor cell lines

We investigated the reactivities of CLN-IgG to the various human tumor cell lines and non-tumorigenic cells by ELISA (Fig. 3). IM-9 IgG was used as an irrelevant, isotype-matched immunoglobulin. CLN-IgG reacted with cervical carcinomas (HeLa229, CaSki and ME180) which were histologically of the same origin as the tumor of the lymphocyte donor. CLN-IgG also bound to other types of tumors such as glioblastoma (U-373MG), stomach carcinoma (AGS), melanoma (G361), prostate carcinoma (PC-3) and lung carcinoma (A549). In contrast, no significant reactions were observed in WI38, MRC-5, MRC-9 and Detroit 551, which are considered to be non-tumorigenic fibroblasts, and in peripheral blood lymphocytes and any types of red blood cells from normal adults. IM-9 IgG was negative to any types of cells (Fig. 3).

Immunofluorescence detection of the antigens recognized by CLN-IgG

The broad reactivity of CLN-IgG with tumor cells was further confirmed by indirect immunofluorescence staining. The formaldehyde-fixed cells were incubated with CLN-IgG and stained with FITC-conjugated anti-human IgG. CLN-IgG reacted with cervical, stomach and prostate carcinoma, but not with fibroblast (Figs. 4, 5).

The antigens recognized by CLN-IgG seemed to be localized in the restricted areas of the positive

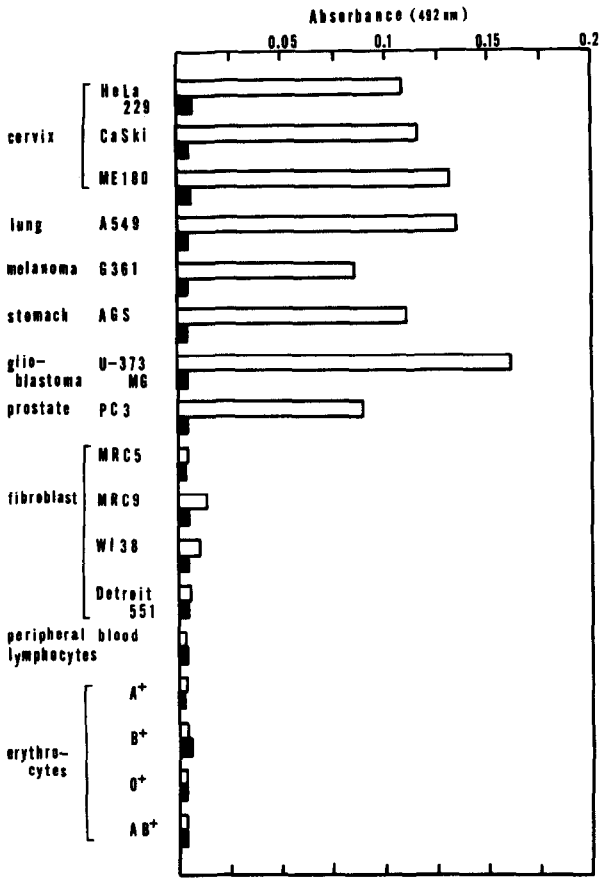

Fig. 3. Reactivity of CLN-IgG to the various human established cell lines and hematopoietic cells measured by ELISA. The data represent the mean values of triplicate determinations. Open bar, CLN-IgG; filled bar, IM-9 IgG.

cells, as indicated by white arrows in Figs. 4 and 5. In CaSki (Fig. 4c) and G361 (Fig. 4d), the tumor cell antigens appeared to be close to the plasma cell membrane; in PC-3 (Fig. 4a), MKN45 (Fig. 4b) and ME180 (Fig. 5a), tumor cell antigens seemed to be exposed towards the outside of the cell.

In addition, all of the ME180 tumor cells were not positive for the antigens detected by CN-IgG immunologically. This result showed the heterogeneity of the cells with regard to the tumor cell antigen expression. Such heterogeneities were similarly observed in PC-3, MKN-45, G361 and CaSki.

Because formaldehyde fixation makes tumor cells permeable to antibodies, it is difficult to determine whether antigens recognized by CLN-IgG are exposed on the cell surface or not. Therefore, we fixed the cells with paraformaldehyde and prepared CLN-IgG-immobilized fluorescent beads to locate the reaction sites of the cells. These MoAb-coated beads were too large to enter the fixed cells. As shown in Fig. 6, the beads bound to the surface of HeLa229 (Fig. 6a) and PC-3 (Fig. 6b) at one limited site. Many beads bound to a glioblastoma cell line U-373MG (Fig. 6c). Binding of the beads to the fibroblast cell line WI38, which showed negative reaction as examined by ELISA and indirect immunofluorescence staining, was not observed (Fig. 6d). These results suggested that the tumor cell antigens could be recognized from the outer surface of the cell by CLN-IgG.

Properties of the antigen purified by affinity chromatography

We purified the tumor cell antigen to homogeneity by using CLN-IgG coupled Sepharose 4B from plasma membrane of A549 lung carcinoma cells. A purified native antigen migrated with a relative molecular mass (M_r) of 226,000 (TA226K) in SDS–polyacrylamide gels, whereas reduction with β-mercaptoethanol generated two polypeptides of M_r 60,000 (α subunit) and M_r 53,000 (β subunit) (TA60K/53K) (Fig. 7A). When irrelevant IM-9 IgG-coupled Sepharose 4B was used, no bands were detected.

To determine whether the same tumor cell antigen was detected on cultured tumor cells, we purified antigens by the affinity chromatography from the plasma membranes prepared from HeLa229, G361, MKN-45 and A549 cells cultured *in vitro*. The TA60K/53K was detected in every tumor cell examined. These data suggest that the TA60K/53K is an antigen commonly expressed by tumor cells of histologically different origins.

DISCUSSION

The human monoclonal antibody, CLN-IgG used in this report was secreted from a human × human hybridoma, termed CLNH11. This hybridoma was generated by fusing lymph node lymphocytes of a cervical carcinoma patient with B-cell lymphoblastoid cell line UC729-6 [10]. Although UC729-6 is known to produce small amount of IgM having κ light chain [9], SDS–PAGE showed that CLN-IgG has single kinds of heavy and light chains. With respect to the monoclonality of the light chain, we ascertained the partial N-terminus amino acid sequence of CLN-IgG (data not shown).

Hagiwara and Sato reported the reactivity of CLN-IgG with tissues and primary cultures of autologous as well as allogeneic cervical carcinomas [10]. Here we re-examined the reactivity of CLN-IgG more in detail using frozen tissue sections, established cell lines and normal hematopoietic cells of the human by means of immunoperoxidase staining, ELISA and immunofluorescence staining. The results showed that, although CLN-IgG was generated from a patient with cervical carcinoma it bound to various tumor cells with histologically different origins. Thus, this antibody was not cervical tumor-specific. Furthermore, CLN-IgG was preferentially reactive to malignant tumor cells because it had no reactivity to non-tumorgenic tissues and cells.

We purified the antigen by affinity chromato-

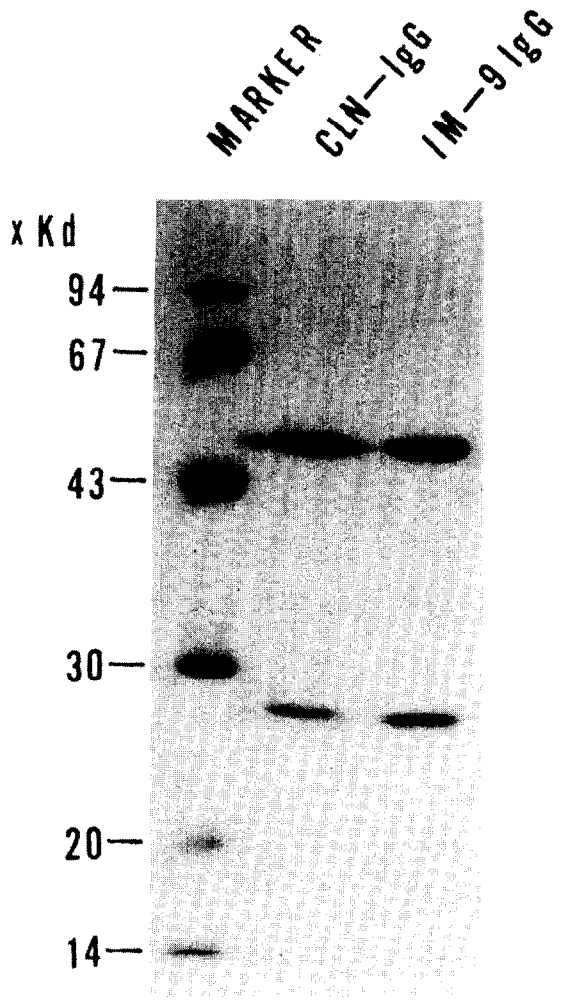

Fig. 1. SDS-PAGE of CLN-IgG and IM-9 IgG. Affinity-purified CLN-IgG (20 μg) *and IM-9 IgG* (20 μg) *were reduced with* 5% β-*mercaptoethanol, analyzed on* 10% *polyacrylamide gel electrophoresis, and stained with Coomassie brilliant blue. Numbers represent molecular weights of marker proteins in kilodaltons. H, heavy chain; L, light chain.*

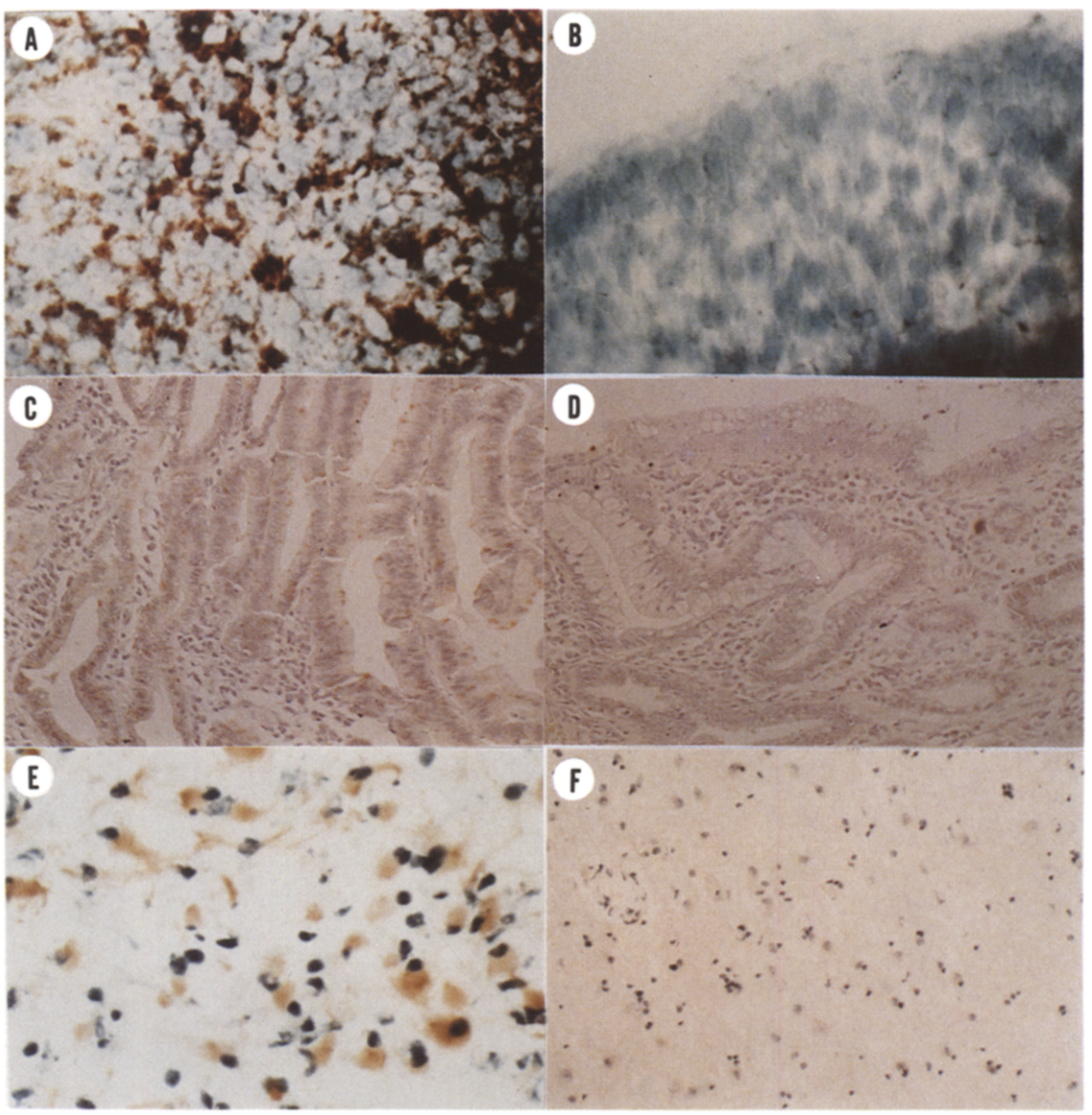

Fig. 2. Immunoperoxidase staining of human frozen tissue sections with biotinylated CLN-IgG. (A) Cervical carcinoma (× 320), *(B) normal cervix* (× 320), *(C) gall bladder cancer* (× 80), *(D) normal gall bladder* (× 80), *(E) glioblastoma* (× 320), *(F) normal brain* (× 80).

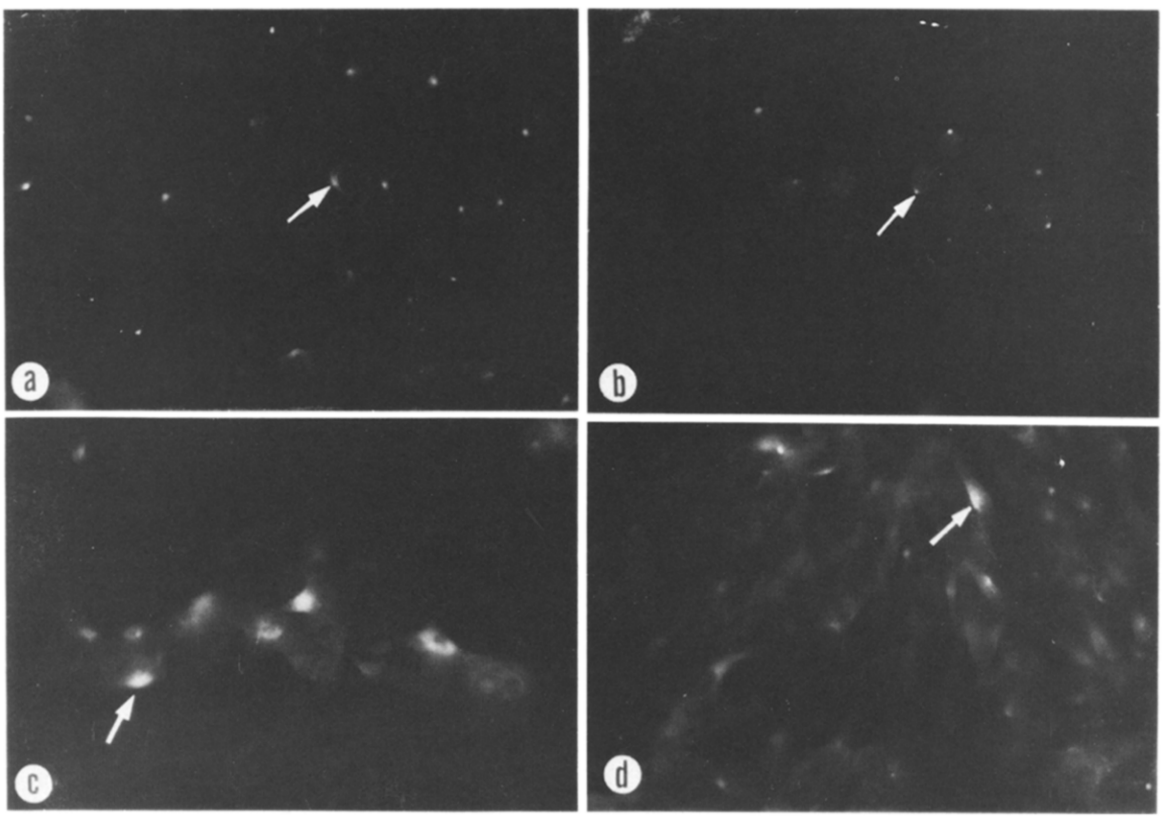

Fig. 4. Indirect immunofluorescence staining by CLN-IgG. (a) PC-3 (prostate carcinoma, × 90), (b) MKN-45 (stomach carcinoma, × 90), (c) CaSki (cervical carcinoma, × 180), (d) G361 (melanoma, × 90). A typical distinctive reaction site is indicated by a white arrow in each tumor cell.

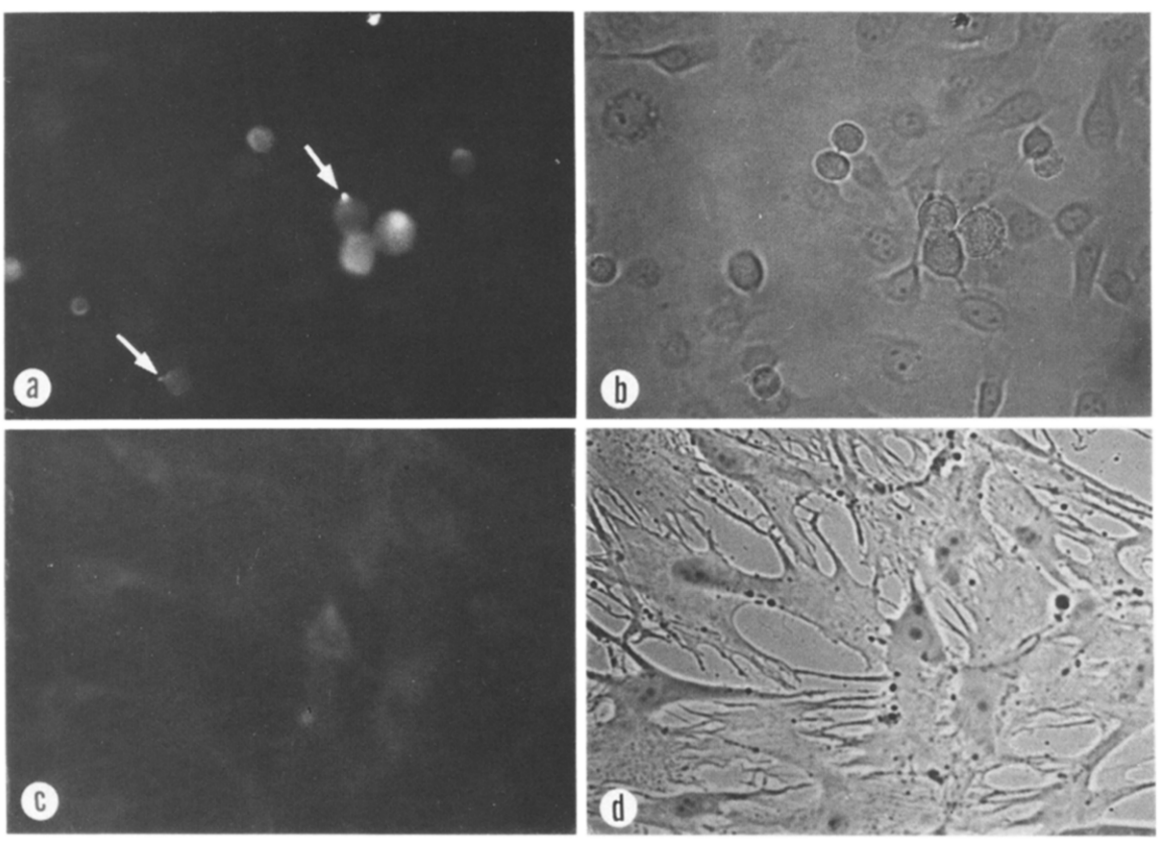

Fig. 5. Indirect immunofluorescence showing heterogeneous expression of CLN-IgG-recognized antigen. (a) ME180 (cervical carcinoma, × 90), (b) phase contrast micrography of the same field as (a), (c) WI38 (fibroblast, × 180), (d) phase contrast micrography of the same field as (c). White arrows indicate the typical distinctive reaction sites.

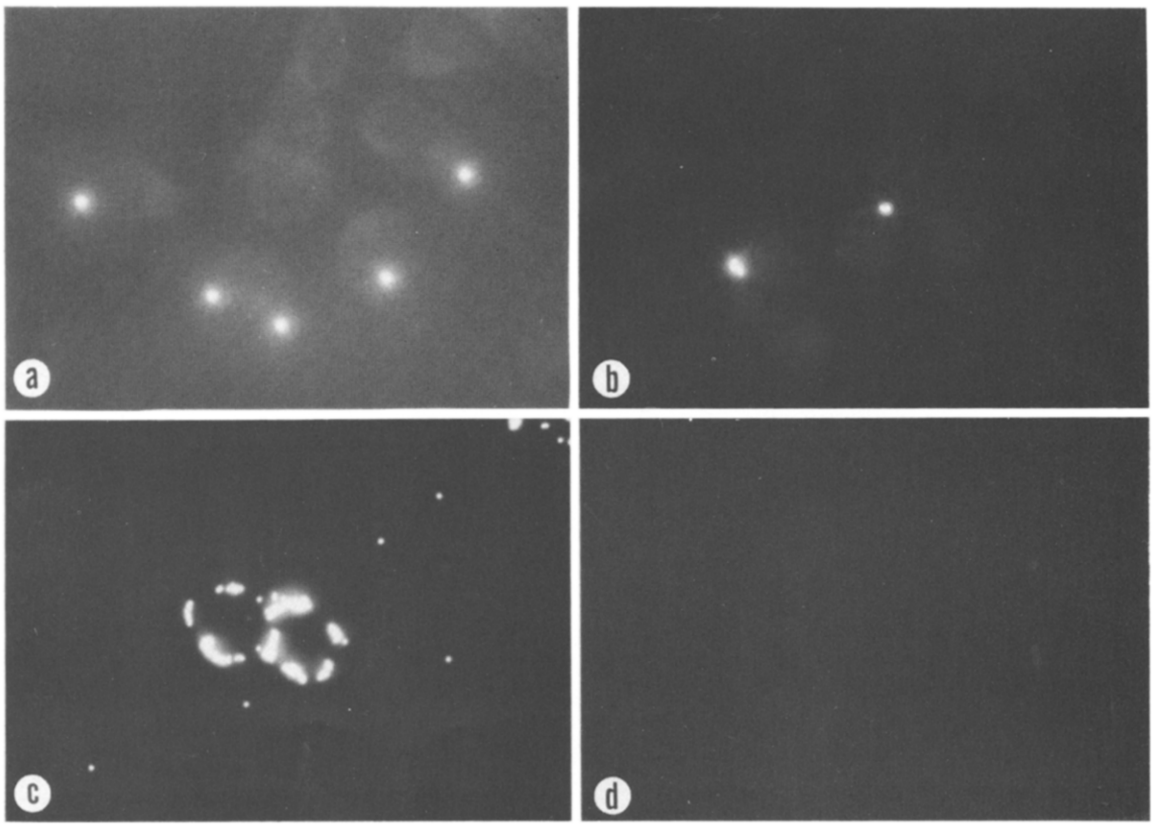

Fig. 6. Direct immunofluorescence staining by using CLN-IgG conjugated fluorescent beads. (a) HeLa229 (cervical carcinoma, × 180), five positive cells were represented. (b) PC-3 (prostate carcinoma, × 360), two positive cells were represented. (c) U373-MG (glioblastoma, × 360), two positive cells were represented. (d) WI38 (fibroblast, × 180).

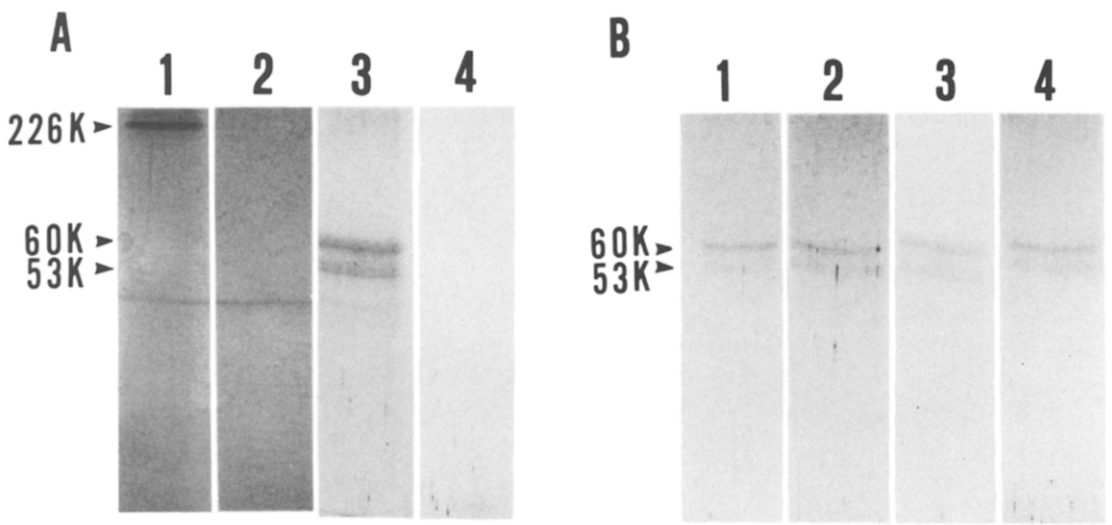

Fig. 7. SDS–PAGE of purified tumor antigen. (A) Antigens were purified from plasma membrane of A549 lung carcinoma grown in nude mice by using CLN-IgG- [1, 3] or IM-9 IgG-conjugated [2, 4] Sepharose 4B; lanes 1 and 2 were non-reduced, while lanes 3 and 4 were reduced by 5% β-mercaptoethanol. The antigens were developed by silver staining. (B) Antigens were purified from plasma membranes of human established cell lines. 1, HeLa229; 2, MKN-45; 3, G361; 4, A549. All samples were reduced with 5% β-mercaptoethanol. The antigens were developed by silver staining.

graphy using CLN-IgG-coupled Sepharose 4B from the membrane fraction of the four human tumor cell lines. SDS–PAGE showed that the antigen had a molecular weight of 226,000 consisting of α (M_r 60,000) and β subunits (M_r 53,000) which were linked by intermolecular disulfide bond(s).

Recently, Feizi reviewed tumor antigens recognized by MoAbs [20]. Most of the MoAbs cited were derived from lymphocytes from mice or rats which had been hyperimmunized previously with human tumors or their extracts. Such MoAbs mainly identified carbohydrate structures of glycoprotein or glycolipid present in many types of cells. Irie *et al.* reported that a human MoAb, secreted from EB-virus transformed human lymphocytes, recognized G_{M2} and G_{D2} glycolipid [21, 22].

In contrast, our results indicate that the epitope of TA60K/53K recognized by CLN-IgG does not seem to be carried on a carbohydrate, because the reactivity of CLN-IgG to tumor cells was resistant to sodium periodate and other deglycosylation treatments.

For the therapeutic application of monoclonal antibodies against cancer, cell surface distributions of the tumor antigens would be crucial, because initial steps of antibody-mediated mechanisms against tumor, such as complement dependent cytotoxicity (CDC) and antibody dependent cell mediated cytotoxicity (ADCC), occur at the surface of tumor cells with the exposed antigens.

Direct and indirect immunofluorescence staining showed that the antigens recognized by CLN-IgG distributed on the regions close to the cell surface membrane and some antigens were exposed on the cell surface. This finding was also supported by the following results: (1) CLN-IgG showed ADCC against cervical carcinoma cell lines (data will be published elsewhere) and (2) the mixture of the CLN-IgG and a different human monoclonal antibody, SLN-IgG, demonstrated synergistic augmentation in CDC activity against glioblastoma cells [23].

The function of the TA60K/53K molecule remains elusive at present, but immunohistological examination of astrocytoma tissues showed that the level of the antigen expression correlated with their malignancies (personal communication with Dr. Taomoto of Kobe University). Therefore, it is conceivable that TA60K/53K may be involved in aberrant behaviors, which are exhibited by tumor cells, for example abnormal growth, metastasis and tissue invasion.

We demonstrated here that human monoclonal antibody technology could provide a new tool to purify and identify novel antigens from human tumor cells. We also showed that cellular components in tumor cells could become immunogenic in the tumor-bearing host. The knowledge of the antigens and antibodies will help to elucidate the tumor genesis and humoral immune response against cancer in humans.

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