主 論 文
子宮頚癌に対するヒト型モノクローナル抗体 [1-1-2D]
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Human Monoclonal Antibody [1-1-2D] Against Cancer of the Uterine Cervix

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ABSTRACT

To obtain a human monoclonal antibody (h-MAb) against cancer of the uterine cervix, lymphocytes from the regional lymph nodes of 14 patients with cervical cancer were fused with a mouse–human heterohybridoma [II]. Of 6,419 hybridomas, 1,295 produced human immunoglobulins (IgG 670, IgM 737). We isolated clone [1-1-2D], which has produced human IgM with stability for more than a year. This antibody reacted with three of five cell lines of cervical cancer but not with normal fibroblasts. Histoimmunostaining showed positive responses to 9/15 specimens of cervical cancer and in 2/7 specimens from cases of cervical dysplasia. Most of the normal human and fetal tissues showed no positive immune response. The positive immune response of [1-1-2D] to the cell membrane observed by the fluorescence antibody method disappeared after periodate treatment and was weakened by trypsin. Neuraminidase did not affect immune reactivity. This antibody showed a positive response by the thin-layer chromatography immunostaining method and precipitated a glycoprotein having a molecular weight of 38 kD. These results suggested that the epitope of the [1-1-2D] antibody is present on a carbohydrate moiety not containing sialic acid that is carried on protein and lipid moieties.

INTRODUCTION

THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES (h-MAbs) against cancer has been increasingly reported in gynecology[1–3] and other fields of medicine.[4] Most of these h-MAbs have not been useful for diagnostic and therapeutic purposes because it is difficult to produce a hbyridoma that stably produces h-MAbs.[5]

We previously established a mouse–human heterohybridoma [II][6] by fusion of the mouse myeloma cell line NS-1[7] and human fetal splenic lymphocytes. This heterohybridoma is an immunoglobulin nonsecreting type with five human chromosomes that are stably retained, and that has a doubling time 20 hr. It is resistant to 8-azaguanine and sensitive to HAT medium. It is a useful parental cell line for producing h-MAb.

We constructed a triple hybridoma that secreted cervical cancer-specific h-MAb [1-1-2D] by cell fusion technique using lymphocytes from regional lymph nodes obtained from patient with cervical cancer and the parental cell line [II]. The development and the characterization of this antibody is described.

MATERIALS AND METHODS

Cell fusion

Lymphocytes were prepared from the removed lymph nodes of 14 patients with cancer of the uterine cervix and cultured for 4 days in RPMI-1640 medium with 10% fetal bovine serum (FBS) and containing 20 μg/ml pokeweed mitogen (PWM).[8]

Cell fusion was performed as follows: mouse–human heterohybridoma [II] and the prepared lymphocytes were mixed at a ratio of 1:1–5, and suspended in a medium consisting of 0.25 M mannitol, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM Tris-HCl pH 7.2. These cells were electrofused in an apparatus (SSH-1, Shimazu, Tokyo, Japan) according to the method of Sugiyama *et al.*[6] Fused cells were seeded and selected in HAT medium.

Detecting the secretion of human immunoglobulin

Human immunoglobulins (h-Igs) in the culture supernatant of the triple hybridoma colony were examined by an enzyme-

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linked immunosorbent assay (ELISA) according to the method of Voller.[9] In brief, 96-well plates (Nunc, Roskilde, Denmark) were coated with goat anti-h-IgG (Tago, Burlingame, CA) or with goat anti-h-IgM (Tago). Culture supernatants were incubated in the plates for 20 hr at 4°C. Then, horseradish peroxidase (HRP)-labeled goat anti h-IgG (Tago), for HRP-labeled goat anti h-IgM (Tago) were added, and the absorbance was determined with phenylenediamine (o-PDA) at 492 nm (Hitachi Type 650-40 spectrophotometer, Tokyo, Japan). A series of diluted h-IgG or h-IgM immunoglobulin-positive controls were used for determination of the h-Ig titer.

Hybridoma selection by reactivity with cancer cells

The reactivity of a culture supernatant of the h-Ig-producing triple hybridoma against five kinds of cervical cancer cells or two kinds of normal human fibroblasts was examined by the Cell-ELISA method[10,11] to select of tumor-specific h-Ig. Each of these cell lines was seeded in a 96-well ELISA plate. After 24 hr of incubation, cells were fixed with 1% glutaraldehyde for 1 hr at room temperature. Absorbency was determined by the ELISA method stated in the preceding section.

Cloning

Triple hybridomas that produced highly tumor-specific h-Ig were immediately monocloned by the limiting dilution method.[12]

Selection of h-MoAB [1-1-2 D]

Hybridomas that produced h-Ig levels of more than 5 μg/ml after cloning were selected by the ELISA method. Following examination of the reactivity to human cell lines and tissue slices, a stable clone [1-1-2D] was isolated.

Reactivity of h-MoAB [1-1-2D]

Reactivity of [1-1-2D] with various tumor cell lines and with normal fibroblast cell lines were examined by the indirect immunofluorescence staining method.[13] Viable cultured cells spread in a single layer were fixed on cover glasses and reacted with [1-1-2D] culture supernatant and FITC-labeled goat anti-h-IgM (Tago). Normal h-IgM (Tago) served as a negative control.

Frozen or formalin-fixed tissue sections from patients with cervical cancer or cervical dysplasia were tested for their reactivity using the immunoperoxidase staining (ABC method),[14] using biotin-goat anti-h-IgM (Tago) and avidin–biotin complex (10 μl of streptoavidin, 10 μl of biotin-HRP, and 1 ml of 1% BSA/PBS) (Dako, Kyoto Japan), and colored with diaminobenzidine. Reactivity with normal human tissues and fetal tissues was also examined. Tissues were obtained from fetuses of gestational ages 10, 11, and 16 weeks. All procedures were performed under in accordance with Japanese eugenic law and the law of anatomy and preservation of cadavers, and were approved by the medical ethics committee of Japan Society of Obstetrics and Gynecology.

Examination of antigens recognized by h-MoAB [1-1-2 D]

Periodate Treatment[15] and Enzyme Treatment[16]: Cells of the HeLa cervical cancer cell line were fixed with 10% neutrally buffered formalin solution for 10 min at room temperature, and treated by one of the following three methods: with 5 mM periodic acid/PBS at 37°C for 10 min,[15] with 0.03% trypsin/PBS at 37°C for 10 min, or with 5 U/ml neuraminidase/10 mM acetate buffer solution pH 6.5 at 37°C for 1 hr.[16] Changes in reactivity of [1-1-2D] were examined by the immunofluorescent method.[13] As the negative control, PBS was incubated at 37°C for 10 min, or 10 mM acetate buffer solution pH 6.5 was incubated at 37°C for 1 hr.

Immunoprecipitation Method[17]: For immunoprecipitation, HeLA cells (2 × 10^7) were incubated in RPMI-1640 containing 0.5 mCi of [^{35}S]methionine (Amersham, Bucks, UK) for 5 hr. Cells were harvested and washed with PBS containing 2 mg/ml of nonradioactive methionine. After protein extraction (1.0% Nonidet P-40, 0.1 mM PMSF, 0.15 M NaCl, 20 mM Tris, and 20 μg/ml aprotinine) at 4°C for 30 min, the mixture was centrifuged at 15,000 rpm for 30 min, and the supernatant was collected. This detergent extract was precleaned with goat anti-human IgM (Tago) to avoid nonspecific reaction. Next, 40 μl of [1-1-2 D] supernatant (10 μg/ml) was added to the supernatant containing 1 × 10^7 cpm of ^{35}S, and allowed to react at 4°C for 24 hr. After addition of 200 μl of goat anti-human IgM (Tago), the mixture was allowed to stand at 4°C for 24 hr. As a negative control, 40 μl of normal human IgM (Tago) (10 μg/ml) was used. After centrifugation in the washing solution (0.25% Nonidet P-40, 0.1% BSA, 0.1% mM PMSF, 0.15 M NaCl, and 20 mM Tris), the pellet was suspended in the sample buffer (200 mM Tris-Cl, 30% glycerol, 10% SDS, 5 mM EDTA, and 6% 2-ME), and subjected to 12.5% SDS-PAGE[18] (Mini Protean II, Bio-Rad, Richmond, CA). The gel was fixed with 10% acetic acid and 25% 2-propanol overnight at room temperature. It was reacted with EN^3 HANCE (New England Nuclear, Boston, MA) at room temperature for 1 hr, dried, and exposed at −80°C for 24 hr.

Thin-Layer Chromatography Immunostaining: HeLa cells (2 × 10^7) were suspended in 0.4 ml of chloroform methanol (2:1, vol/vol) for 3 hr for extraction. The mixture was centrifuged at 12,000 rpm for 5 min, and 3 μl of the supernatant was subjected to thin-layer chromatography (TLC). We used a silicagel plate (Polygram, Macherey-Nagel Co., Germany), with a developing solvent mixture of chloroform/methanol/0.02% $CaCl_2$-H_2O (60:35:8, vol/vol). After the TLC plate was air-dried, blocking was performed with 1% ovalbumin–1% polyvinyl pyrrolidone (Sigma, St Louis, MO)–FBS solution at room temperature for 1 hr. Next, [1-1-2 D] supernatant was added to the mixture and reacted at room temperature for 30 min. After washing with 0.1% Tween 20–FBS solution, HRP-labeled goat anti-human IgM (Tago) (×1,000) was added, and the mixture reacted at room temperature for 30 min. After washing with PBS, it was stained with DAB/$NaCl_2$. Normal human IgM (Binding Site Inc.) was used as the negative control.

RESULTS

Cell fusion and selection of [1-1-2 D]

As shown in Table 1, 3.58 × 10^8 lymphocytes were obtained from 14 patients with cervical cancer. After cell fusion using an electric pulse, cells were seeded into 8,208 wells. The number of

TABLE 1. CELL FUSION WITH REGIONAL LYMPHOCYTES FROM 14 CASES CERVICAL CANCER AND HETEROHYBRIDOMA [II]

Patient number	*Stage (FIGO[a])*	*Lymphocytes[b] (×106)*	*Wells with viable hybridoma[c]*	*Ratio viable hybridoma[d]*	*Wells with h-Ig-producing hybridoma*	*Ratio h-Ig-producing hybridoma[d]*
1	Ib	10	288/ 288	28.8	36	3.6
2	Ib	13	259/288	19.9	89	6.8
3	Ib	7.5	321/336	42.8	59	7.9
4	Ia	40	828/960	20.7	55	1.4
5	IIb	24	526/768	21.9	21	1.0
6	Ib	8.8	156/192	17.7	5	0.6
7	O	2.2	15/96	6.8	1	0.5
8	O	9.2	83/384	9.0	10	1.1
9	IIa	4	53/96	13.3	2	0.5
10	IIa	2.4	18/96	7.5	0	0
11	Ib	3.3	91/96	27.6	22	6.7
12	Ib	67	1,267/1,440	18.9	424	6.3
13	Ib	12	239/288	19.9	22	1.6
14	Ib	155	2,275/2,880	14.7	549	3.5
Total		358.4	6,419/8,208	17.9	1,295 IgG 670 IgM 737	3.6 IgG 1.87 IgM 2.06

[a]International Federation of Gynecology and Obstetrics
[b]No. of lymphocytes after pokeweed mitogen stimulation
[c]No. of wells with viable hybridomas/No. of wells seeded
[d]per 10^6 lymphocytes

wells with viable hybridomas was 6,419 (78.2%). The ratio of viable hybridomas was 17.9 per 1×10^6 lymphocytes. The number of human Ig-producing hybridomas was 1,295. Human IgG production was found in 670 wells and human IgM production in 737 wells, with 112 wells producing both immunoglobulins.

The reactivity of the triple hybridoma supernatants with more than 0.1 μg/ml titer was examined using cervical cancer (A) and normal human fibroblast (B) cell lines. Results were categorized in four groups: positive for both A and B, positive for A but negative for B, negative for both A and B, and indistinct, with ratios of about 10:5:3:7, respectively. The positive A and negative B groups were considered to be possibly cancer-specific, cloning was performed for 55 colonies in these groups.

After cloning, 36 clones maintained the production of h-Ig of more than 5 μg/ml titer. Five colonies reacted with unfixed viable cells by the fluorescence antibody method. The reactivity of these 5 colonies with cervical cancer tissues was examined and human MoAB [1-1-2D] was selected.

Immunospecificity of [1-1-2D]

Reactivity with Various Cancer Cell Lines: As shown in Table 2, three of the five lines of cervical cancer cells reacted positively with [1-1-2D], while the two normal human fibroblast cell lines did not. Some of the cell lines from cases of uterine corpus cancer, ovarian cancer, and gastric cancer showed positive reactivity to [1-1-2D].

Reactivity with Uterine Cervical Tissues: Table 3 indicates the reactivity of [1-1-2D] to normal uterine cervical squamous epithelium, dysplastic and cancerous tissues. Of 7 cases of normal squamous epithelia, 1 showed a positive reaction, while 6 cases showed not staining. Cases of mild dysplasia reacted negatively, while 1/2 cases of moderate dysplasia reacted positively. Severe dysplasia showed positivity in 1/2. Carcinoma *in situ* showed a positive reaction in 2/4, and invasive cancer in 7/11 cases. The positive reaction in an invasive cancer is seen around the cancer cell membrane in Figs. 1, A and B. Figure 1, C and D, also show positive staining. Figure 2, A and B, show positive staining of the normal squamous epithelia. The positive reaction extends from the basal layer to the parabasal layer. These findings in Figs. 1 and 2 were negative when normal human IgM was used as the control specimen.

Reactivity with Normal Tissues and Fetal Tissues: [1-1-2D] showed weak staining (not shown) in some specimens of normal esophagus (1/2), spleen (1/3), renal tubule epithelium (1/2), and skin (1/2), but not in others: lung (0/1), heart (0/1), mammary gland (0/1), stomach (0/3), colon (0/1), liver (0/1), pancreas (0/1), ovary (0/3), vagina (0/5). No reaction was observed in specimens of fetal tissue: heart (0/2), lung (0/2), liver (0/3), spleen (0/1), stomach (0/1), colon (0/3), kidney (0/1), skin (0/2), bone (0/1).

Examination of antigen recognized by human MoAb [1-1-2D]

Immunofluorescence staining of HeLa cells with [1-1-2D] antibody showed positive reactivity at the cell-surface membrane (Fig. 3). This reactivity was weakened by trypsin treatment, and disappeared after periodate treatment, but was unchanged by neuraminidase treatment. A band was detected at 38 kD by immunoprecipitation method (Fig. 4). A positive spot was observed on thin-layer chromatographic immunostaining (Fig. 5).

TABLE 2. REACTIVITY OF [1-1-2D] ANTIBODY WITH VARIOUS CANCER CELL LINES

Tumor	*Cell line*	*Reactivity*[a]	*Tumor*	*Cell line*	*Reactivity*[a]
Uterine cervical cancer			Lung cancer		
Squamous cell carcinoma	SiHa	−	Adenocarcinoma	A549	−
	ME180	−	Squamous cell carcinoma	QG56	−
	AMCC	++	Small cell carcinoma	QG90	−
Adenocarcinoma	HeLa	++	Large cell carcinoma	IMAI	−
	TMCC	++	Stomach cancer	NUGC 3	−
				NUGC4	+
Uterine corpus cancer	HEC 1A	−	Colon cancer	SW1083	−
	AMEC	++	Hepatic cancer	Epstain	−
Ovarian cancer					
Serous adenocarcinoma	SKOV	−	Kidney cancer	Scattla	−
	NOS 3	−		Brakatzky	−
	NOS 4	++	Normal fibroblast	MRC-5	−
	NOS 1	++		F 7000	−
	NOS 2	−			
Mucinous adenocarcinoma	NOM 1	+			
	NOM 2	−			

[a] ++, More than half of cells positive; +, less than half of cells positive; −, negative.

These findings suggested that the antigen recognized by the [1-1-2D] antibody is a carbohydrate moiety that does not contain sialic acid and is carried on glycoprotein and glycolipid.

DISCUSSION

Routine screening for cancer of the uterine cervix has improved the ability to detect and treat early disease. However, the management of progressive or recurrent cervical cancer requires new methods of diagnosis and treatment. Mouse MAb has been used in the diagnosis[19,20] and treatment[21] of gynecological cancers. However, the production of anti-mouse antibody[22] by the patient may weaken the therapeutic effect and produce allergic reactions. Chimeric antibody (human-mouse MAb) has been produced and administered to human subjects[23]; however, this antibody may also indice anti-mouse antibody.[24] Human MAb should avoid these problems.

Of the four procedures for producing human MAb, three have disadvantages: The Epstein-Barr (E-B) virus transformation method[25] may give only a small amount of antibody,[26] and there may be a risk of contamination with E-B virus; the human–human cell fusion method[27] has a low fusion rate; and mouse–human cell fusion[28] results in the loss of human chromosomes.[29] For these reasons, we explored another method, the mouse–human–human triple hybridoma method for producing h-MAb,[3] and successfully obtained a stable human MAb-producing cell line, using mouse–human heterohybridoma [II].

For the cell fusion process, we avoided using polyethylene glycol[31] because of cytotoxicity. With the electric fusion method,[32] we obtained a large number of viable cells and a high fusion rate. The method may be used for relatively small numbers of cells, is easy to perform, gives satisfactory results, and is reproducible. The rate of colony formation in this study was 17.9 per 1×10^6 lymphocytes, and human Ig-producing colonies were 3.6 per 1×10^6 lymphocytes. However, in some patients, the rate of cell fusion was not effective, and when lymphocytes were less than 5.0×10^6, the rate of cell fusion was lower.

Human MAb [1-1-2D] obtained in our study is the IgM type, as reported for most tumor-specific human MAbs. Komisar *et al.* reported that E-B transformation prior to cell fusion, made it easier to obtain IgM-producing hybridomas.[33] We found that the IgM-producing hybridomas were slightly more formed than the IgG-producing hybridomas, using triple hybridoma method.

The [1-1-2D] antibody showed some reactivity to cancer cells, other than those from uterine cervical cancer, indicating

TABLE 3. REACTIVITY OF [1-1-2D] ANTIBODY TO UTERINE CERVICAL TISSUES

	Normal cervix	*Dysplasia*			*Uterine cervical carcinoma*	
		Mild	*Moderate*	*Severe*	*CIS*	*Invasive*
Number tested	7	3	2	2	4	11
Number of positive	1[a]	0	1	1	2	7
Ratio of positive (%)	14	0	50	50	50	64
			29 (2/7)		60 (9/15)	

CIS, Carcinoma *in situ*.
[a] Positive from the basal layer to the para basal layer.

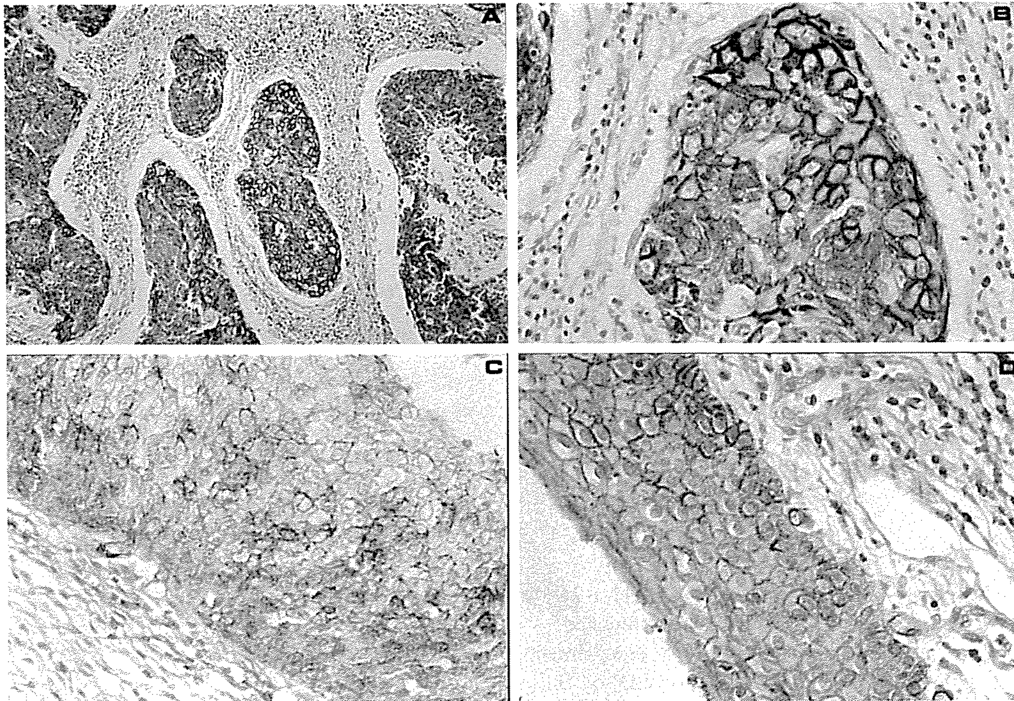

FIG. 1. Immunoperoxidase staining of uterine cervical tissues by [1-1-2D] antibody counterstained with methylgreen. One case of invasive cancer (A,B), severe dysplasia (C), and moderate dysplasia (D) show strong staining around the cell membrane (A, 25×; B, 100×; C, 100×, D, 100×).

that the antibody is not strictly specific for one type of cancer cell, but may react to other cancer-related antigens. The [1-1-2D] antibody may recognize the carbohydrate moiety of a glycolipid. Since the reactivity of this antibody was weakened by trypsin treatment, and a 38-kD band was found on immunoprecipitation, this carbohydrate moiety may be also present as a glycoprotein. An additional band sometimes appeared in lane 2 (Fig. 4). It is not clear what this means. The antigen recognized by the [1-1-2D] antibody needs further investigation.

This is the first report of the production of a h-MAb against uterine cervical squamous cell carcinoma. Previously, a h-MAb was obtained from the lymphocytes of patients with uterine cervical adenocarcinoma.[2] The reactivity of that antibody with malignant glioma may be useful in the field of cerebral nerve surgery.[34] The reactivity of [1-1-2D] with other malignant tumors should be studied.

The clinical application of h-MoAB has been attempted in diagnostic imaging[35,36] and cancer therapy[37,38] because of its high selectivity and low side effects. Since [1-1-2D] recognizes a cell membrane antigen, it may be favorable for accumulation on the tumor. Because of its IgM type, however, it may not be suitable for use as a radioisotope or drug carrier. A class-

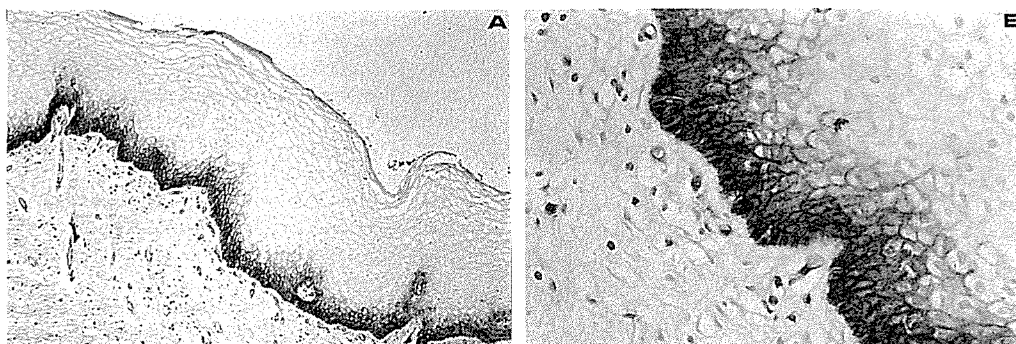

FIG. 2. Immunoperoxidase staining of normal cervical tissue sections by [1-1-2D] antibody. There is positive staining from the basal to the parabasal layer. (A, 25×; B, 100×).

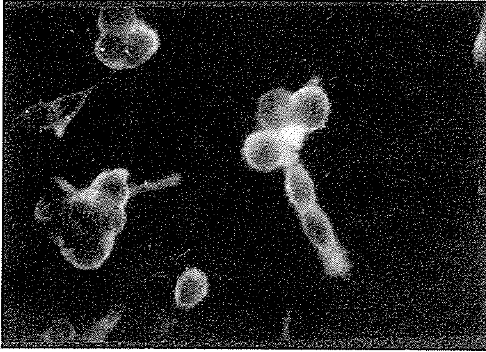

FIG. 3. Immunofluorescence staining of HeLa cells with [1-1-2D] antibody shows a positive reaction consistent with the cell surface membrane (400×). Such reactivity disappeared after periodate treatment.

subclass switch method(39) may be used to alter the [1-1-2D] to the IgG type, and a more favorable MAb may be produced.

The IgM type antibody generally exerts a complement-dependent cell toxicity, and this is true with [1-1-2D] on HeLa cell lines *in vitro* (data not shown). Irie *et al.* used an IgM-type human MAb for treating patients with melanoma, and reported that an IgG-type antibody was induced in the patient's serum.(40) That anti-idiotype treatment may induce an IgG-type antibody and it warrants further study. The clinical application of [1-1-2D] for treating patients with cancer of the uterine cervix merits investigation.

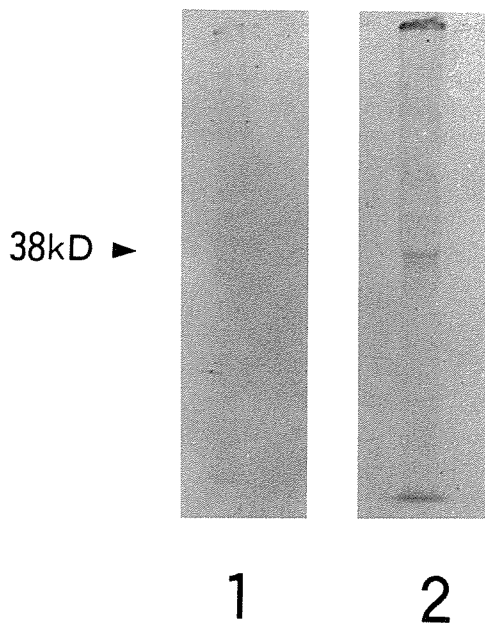

FIG. 4. Immunoprecipitation of [^{35}S]methionine-labeled HeLa cell lysates with [1-1-2D] antibody (lane 2) and normal human IgM (lane 1).

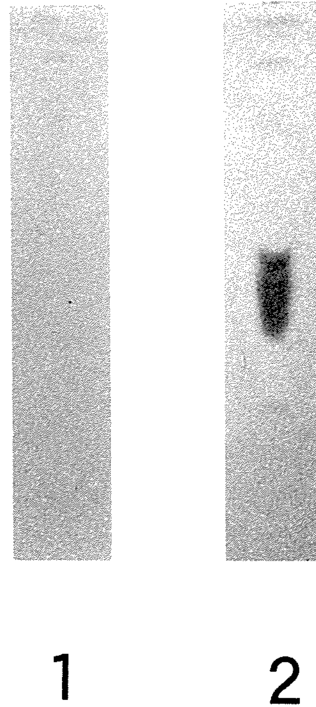

FIG. 5. Thin-layer chromatography immunostaining. There is a positive spot in lane 2 with [1-1-2D] and a negative reaction in lane 1 with normal IgM.

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