Preparation and Characterization of Anti-Anti-Idiotypic Monoclonal Antibody (“Ab1-Like Ab3”) in Relation to Carcinoembryonic Antigen

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In order to analyze the epitope structure of carcinoembryonic antigen (CEA) and the idiotype network system, seven anti-idiotypic monoclonal antibodies (anti-Id MoAbs) were generated from a BALB/c mouse immunized with anti-CEA MoAb P1-356, which recognized a synthetic peptide P1 of CEA, domain I. These MoAbs were divided into four groups. The anti-Id MoAbs specifically reacted with MoAb P1-356, but not with any MoAb, and inhibited the binding of MoAb P1-356 to CEA, indicating that all of these anti-Id MoAbs recognized private idiotopes at the combining sites of MoAb P1-356. Polyclonal anti-anti-idiotypic antiserum (Ab3) generated with anti-Id MoAbs M315 (Ab2), blocked the binding of MoAb P1-356 (Ab1) to CEA and reacted with CEA(Ag) and synthetic

peptide P1. The analysis of serological assays suggested that it contains “Ab1-like Ab3.” Therefore, we prepared anti-anti-Id MoAbs using anti-Id MoAb M315. Among 13 candidates, anti-anti-Id MoAb 11B2 was selected, because it competed with MoAb P1-356 (Ab1) binding to CEA. A direct binding assay using MoAb 11B2 showed that it reacted with purified CEA and with CEA synthetic peptide P1. In addition, MoAb 11B2 (Ab3) reacted with both CEA-producing cultured cells and colonic cancerous tissues in immunostaining. These results indicate that anti-anti-Id MoAb 11B2 is “Ab1-like Ab3.” Therefore, it is suggested that anti-Id MoAb M315 bears an “internal image” of the MoAb P1-356-defined epitope on CEA. *J. Clin. Lab. Anal.* 16:279–289, 2002. © 2002 Wiley-Liss, Inc.

Key words: CEA; monoclonal antibody; anti-idiotypic antibody; internal image; anti-anti-idiotypic antibody; Ab1-like Ab3

INTRODUCTION

The idiotype network theory was first proposed in 1974 (1), and has been accepted as a basic concept of immune regulation. The function of the immune network is to respond to specific stimulation and to maintain a state of balance throughout the response. A classification of anti-Id antibodies has been proposed (2) by which major types of anti-Id antibodies can be distinguished depending on the topography of the target idiotope. If the idiotope to which an anti-idiotope binds is remote from the binding site (paratope) of the idiotype molecule (Ab1), the binding is classified as α -type (Ab2 α). The β -type anti-Id (internal image anti-Id, Ab2 β) is an antibody that expresses an idiotypic determinant that mimics the structure of antigen for Ab1. Therefore, some of the anti-anti-Id monoclonal antibodies (MoAbs) generated from Ab2 β could react to the antigen and play the role of “Ab1-like Ab3.”

Recently, antitumor vaccination using anti-Id MoAbs, which trigger the immune response as the antigenic stimulator and suppress the tumor growth, has been discussed (3,4).

In a previous study (5) we prepared MoAb P1-356 specific to synthetic peptide (P1: 119–140 amino acids) in domain I of carcinoembryonic antigen (CEA) as one of the main tumor markers of human colorectal carcinoma. In the present study, we prepared the anti-Id MoAbs (Ab2 β) bearing an internal image of CEA, and the anti-anti-Id MoAb (Ab3), to investigate the

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internal image from the molecular aspect, and to determine what kind of anti-Id antibody is useful for vaccination or cancer therapy.

MATERIALS AND METHODS

Development of MoAb to Synthetic CEA Peptide P1

MoAb P1-356 (IgG1) was prepared to a synthetic peptide (P1: 119–140 amino acids) in domain I of the CEA molecule, as described elsewhere (5). The MoAbs were secreted hybridomas constructed with myeloma cells and splenocytes from BALB/c mice, which were immunized once a week with three intraperitoneal injections of 100 µg of synthetic peptide P1 suspended in Freund's complete adjuvant. Hybridization with murine myeloma cells P3-X63-Ag8.653 and subcloning were performed according to standard procedures (6). MoAbs from the resulting hybridomas were purified by caprylic acid preparation (7), affinity chromatography on protein A sepharose, or ion exchange chromatography on DEAE-cellulose (8). Antibodies were radiolabeled with ^{125}I , utilizing the chloramine T method (9).

Development of Anti-Idiotypic (Id) MoAb

Anti-Id MoAbs (Ab2) were prepared against murine anti-CEA peptide MoAb P1-356 (Ab1) in a syngeneic system, using procedures described previously (10). Briefly, hybridomas were constructed with splenocytes from a BALB/c mouse primed with 100 µg of purified murine anti-CEA peptide MoAb P1-356 coupled to keyhole limpet hemocyanin and polymerized with glutaraldehyde in CFA (GIBCO Laboratories, Detroit, MI).

Sandwich Assay

The sandwich assay, as described previously (11), was performed as follows: 96-well polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were coated with MoAb P1-356 by incubating 100 µg of a MoAb solution (100 µg/mL) in a 0.1-M bicarbonate buffer, pH 9.5, for 16 hr at 4°C.

After the plates were washed twice with PBS (pH 7.4), 100 µL of hybridoma supernatants were added and incubation was continued for 16 hr at 4°C. The plates were washed three times with PBS, ^{125}I -labeled MoAb P1-356 was added, and the plates were incubated for 3 hr at 4°C. The plates were then washed five times with PBS, the wells were cut, and the radioactivity was counted in a gamma counter.

Inhibition Assay

Fifty µL of ^{125}I -labeled MoAb P1-356 (1×10^5 cpm) were incubated for 2 hr at 4°C with 50 µL of hybridoma supernatants, and 96-well polyvinyl chloride microtiter plates were coated with purified CEA (20 µg/mL) for 16 hr at 4°C. The plates were washed two times with PBS, and the mixture was added to the plates. At the end of the 2-hr incubation at 4°C, the plates were washed five times with PBS, and the bound radioactivity was counted in a gamma counter. Results were expressed as the percentage of binding inhibition as compared with control.

Idiotypic Binding Assay

Various MoAbs were coated in the microtiter plates. After they were washed two times with PBS, 100 µL (2×10^5 cpm/well) of ^{125}I -labeled anti-Id MoAbs were added and incubated for 3 hr at 4°C. The plates were washed five times with PBS, and the bound radioactivity was counted in a gamma counter (3).

Cross Blocking Assay

Microtiter plates were coated with MoAb P1-356 (Ab1) and incubated for 16 hr at 4°C. After they were washed, an excess of cold anti-Id MoAb (Ab2) was added and the plates were incubated for 16 hr at 4°C. After five washes, ^{125}I -labeled anti-Id MoAbs (1×10^5 cpm/well) were added to test for their ability to bind. After incubation for 3 hr at 4°C, the plates were washed five times and bound radioactivity was counted. Results were calculated as the percentage of blocking compared with the negative control (10% fetal calf serum) (12).

Induction of Syngeneic Anti-Anti-Idiotypic Antisera

BALB/c mice were immunized by an intraperitoneal injection of 100 µg of purified anti-idiotypic MoAb coupled to solubilized keyhole limpet hemocyanin by glutaraldehyde fixation and mixed with complete Freund's adjuvant. Mice were bled 3 days after the last booster and their antisera were used in the analysis.

Competition Assay

A total of 50 µL of serial diluted anti-anti-idiotypic sera (Ab3) (1:10, 1:50, 1:250) generated with anti-idiotypic MoAb (Ab2) and 50 µL of ^{125}I -labeled anti-CEA peptide MoAb P1-356 (Ab1, 1×10^5 cpm) were added to CEA-coated plates and incubated for 2 hr at 4°C. The plates were washed five times and the radioactivity was counted. Results were calculated as

the percentage of competition compared with that of normal sera (12).

Binding of Anti-Anti-Id Antisera (Ab3) to Purified CEA

Anti-anti-Id antisera (1/10 dilution) were added to CEA-coated plates and incubated for 4 hr. After three washings, the plates were incubated with ^{125}I -labeled anti-mouse IgG-Fc antibody for 3 hr. The radioactivity was counted after a final washing.

Development of Anti-Anti-Idiotypic MoAbs

Anti-anti-Id MoAbs (Ab3) were prepared against anti-Id MoAb M-315 (Ab2) and M327 (Ab2) in a syngeneic system, using the same procedures as described for the preparation of Ab2.

Binding Activities of Anti-Anti-Id MoAbs to Anti-Id MoAbs

To determine the reactivities of anti-anti-Id MoAbs, a direct binding assay was performed. ^{125}I -labeled anti-anti-Id MoAbs were added to the anti-Id MoAb-coated plate and incubated for 2 hr. After washing, bound radioactivity was counted.

Binding Activities of Anti-Anti-Id MoAb to CEA and to Synthetic CEA Peptides

An indirect binding assay to CEA was performed. Briefly, supernatants of anti-anti-Id MoAbs were added to the purified CEA-coated plate, and incubated for 3 hr at 4°C. After washing, ^{125}I -labeled anti-IgG-Fc antibody was added and then incubated, and the radioactivity was counted. A direct binding assay was performed with the use of ^{125}I -labeled anti-anti-Id MoAbs and plates coated with synthetic CEA peptides (P1; 119–140, P3; 367–386, and P6; 1–20), as described elsewhere (5).

Competition Assay

To investigate the differences in specificity between anti-anti-Id MoAb (Ab3) and anti-synthetic CEA peptide MoAb P1-356 (Ab1), a competition assay was performed. As described above, a total of 50 μL of Ab3 supernatant and 50 μL of ^{125}I -labeled MoAb P1-356 (Ab1) were added to CEA-coated plates and incubated. After the plates were washed, the radioactivity was counted and results were calculated as the percentage of competition.

Immunoperoxidase Technique

A conventional procedure for the immunoperoxidase technique was employed for the staining actions with

MoAbs (13). MKN 45 (gastric cancer), JRST (gastric cancer), and WiDr (colonic cancer), which produce CEA and RPMI 8226 (B lymphoblast) as controls, were used. These cells were centrifuged at 1,500 r.p.m.; they adhered to the glass slides and were fixed by acetone.

Five colonic cancerous tissues were obtained from patients who had undergone surgery. The tissues were fixed with 10% buffered formalin, embedded in paraffin, and sectioned to 4–5 μm in thickness.

The sections of cells and tissues were first incubated with 0.3% H_2O_2 in methanol for 20 min to block endogenous peroxidase, and then overlaid with MoAbs for 30 min at room temperature in a moist chamber. After they were washed three times for 5 min in cold PBS, peroxidase-conjugate rabbit anti-mouse Ig antiserum (Dako, Copenhagen, Denmark) was applied for another 30 min. After a final wash with cold PBS, the sections were reacted for 60 sec in a solution containing 40 mg 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in 100 mL 0.05 M Tris-HCl buffer (pH 7.6). They were stained with 0.2% methyl green for 12–15 hr and were then observed with a light microscope.

RESULTS

Development and Characterization of Anti-Idiotypic Monoclonal Antibodies to Anti-CEA MoAb P1-356

Syngeneic anti-Id MoAbs were prepared to anti-CEA MoAb P1-356. The cell fusion experiments resulted in 16 of 398 (4.0%) clones, whose supernatants showed strong reactivity in sandwich and inhibition assays. Of these, seven MoAbs (designated as M315, M321, M327, M332, M371, A13, and E25) were established and used in this study. The sandwich assay represents the binding activity of anti-Id MoAb to MoAb P1-356, and the inhibition assay demonstrates the inhibitory activity of anti-Id MoAb against the binding of ^{125}I -labeled MoAb P1-356 to CEA. Representative results are shown in Table 1. Seven anti-Id MoAbs screened in this study recognized MoAb P1-356 and inhibited the binding of MoAb P1-356 to CEA.

The specificity of four representative anti-Id MoAbs was determined by direct binding assay with the use of a variety of MoAbs. As shown in Table 2, each anti-Id MoAb to MoAb P1-356 recognized the private idiotype, since it did not react with a panel of MoAbs to distinct epitopes of CEA or to NCA, mucin, high-molecular-weight melanoma associated antigen (MAA), x-hapten, HLA-class antigen, or BSA. It only reacted with whichever corresponding MoAb P1-356 was used as

the immunogen. The results presented in Tables 1 and 2 suggest that anti-Id MoAbs M315, A13, M332, and M327 specifically recognize idiotopes at the combining site of MoAb P1-356.

TABLE 1. Characteristics of anti-Id MoAbs elicited with anti-CEA MoAb P1-356

Ab2	Isotope	Inhibition assay ^a	Sandwich assay ^b
M315	IgG ₁	96%	15800cpm ^c
M321	IgG ₁	97	16100
M327	IgG ₁	73	9300
M332	IgG ₁	68	9800
M371	IgG ₁	54	11700
A13	IgG ₁	88	11200
E25	IgG ₁	89	12800
10% FCS		0	3000

^aThe mixture of ¹²⁵I-labeled MoAb P1-356 and hybridoma supernatants was added to CEA coated plates. After being washed, the bound radioactivity was counted. The results are expressed as percentage of inhibition, compared with that of 10% fetal calf serum (FCS).

^bMicrotiter plates coated with MoAb P1-356 were incubated with hybridoma supernatants. After being washed, ¹²⁵I-labeled MoAb P1-356 was added to test for binding.

^cMean cpm of duplicate values.

IDIOTOPE MAPPING

To analyze the spatial relationships among idiotopes on P1-356, a cross blocking assay was performed with the use of anti-Id MoAbs. As shown in Table 3, anti-Id MoAbs M315 and M321 (a), A13 and E25 (b), M332 and M371 (c), and M327 (d) showed different

TABLE 3. Idiotopes recognized with anti-idiotypic MoAbs to anti-CEA MoAb P1-356 in cross blocking assay

Cold Ab2	¹²⁵ I-labeled anti:Id MoAb						
	M315	M321	M327	M332	M371	A13	E25
M315	97 ^a	97	0	45	42	79	89
M321	98	97	0	43	48	80	75
M327	0	0	94	28	28	18	13
M332	38	41	17	92	93	48	36
M371	43	48	20	93	91	41	40
A13	71	72	17	48	50	94	88
E25	63	65	13	52	49	87	89

^aMicrotiter plates coated with MoAb P1-356 were incubated with an excess of cold anti-idiotypic monoclonal antibodies and then tested for their ability to bind ¹²⁵I-labeled anti-idiotypic MoAbs.

^aThe results are expressed as percentage inhibition.

TABLE 2. Specificity of anti-Id MoAbs elicited with anti-CEA MoAb P1-356*

MoAb	Specificity	¹²⁵ I-labeled anti-idiotypic MoAb			
		M315	M327	M332	A13
P1-356	CEA	21700 ^a	11900	17900	16500
P1-706	CEA	900	1600	1100	1200
P1-234	CEA	1000	1300	1100	1000
P1-123	CEA	1000	1500	1200	1400
MA208	CEA	1100	1900	1400	1100
5B3	CEA	1200	800	1000	1200
010	CEA	1000	2000	1400	1500
CEA281	CEA	1100	1900	1300	1100
T84-66	CEA	1000	2300	1500	1400
CEA41	CEA	900	1900	1100	1200
261.15.1	CEA	800	1800	1200	1100
12.140.7	CEA	900	1600	1400	1200
12.140.4	CEA	1100	2100	1100	1100
YK024	CEA	1100	1700	1200	1200
MT008	CEA	1500	1800	1400	1300
MA503	NCA	1000	1600	1300	1600
MA504	NCA	1100	1800	1400	1200
YH206	Mucin	1000	1800	1100	1200
MUSE11	Mucin	1000	2000	1300	1100
BM 2	X-hapten	1300	2000	1100	1200
225.28	HMW-MAA	1000	2100	1200	1200
CL203	96kd MAA	1100	1600	1200	1100
CL11-351	HLA-A2,A28	900	2300	1300	1200
KSI	HLA-A2,A28	900	2300	1300	1200
BSA		1400	1300	1200	1100

*¹²⁵I-labeled monoclonal anti-idiotypic antibodies were incubated for 3 hours at 4°C with MoAb-coated microtiter plates. Then the plates were washed and bound radioactivity was counted in a γ-counter.

^aResults are expressed as cpm.

Fig. 1. Mapping of idiotopes on anti-CEA MoAb P1-356. There are at least four idiotopes on the paratope of MoAb p1-356.

specificities. Therefore, these results suggest that there were at least four distinct idiotopes (a–d) at the combining site of MoAb P1-356. Considering the percentage of inhibition among groups a–d, a schematic figure is shown in Fig. 1.

Induction and Analysis of Anti-Anti-Idiotypic Antisera

As described above, selected and established anti-Id MoAbs recognized the combining site of anti-CEA MoAb P1-356 and inhibited the binding of P1-356 to

CEA. Therefore, it is possible that some of the anti-Id MoAbs have an “internal image of antigen (CEA).” Immunization with four distinct anti-Id MoAbs (M315, A13, M332, and M327) in the syngeneic system was done to examine whether they could induce anti-anti-Id antibodies. The results summarized in Table 4 show that anti-Id MoAbs (Ab2) induced anti-anti-Id antibodies (Ab3) specifically reactive to the corresponding immunizing anti-Id MoAb, but not to irrelevant MoAbs in the sandwich assay. The induction of anti-anti-Id antibodies specific to corresponding immunizing anti-Id MoAb was also suggested, since anti-Id

TABLE 4. Analysis of anti-idiotypic antisera (Ab3) generated with anti-idiotypic monoclonal antibodies to anti-CEA MoAb P1-356

Assay	Monoclonal anti-idiotypic antibodies (Ab2)	Anti-anti-idiotypic antiserum (Ab3) generated with				Preimmune serum
		M315	M327	M332	A13	
Sandwich	M315	4773 ^a	1627	1931	1570	1420
	M327	716	6563	1493	714	694
	M332	470	475	9431	842	751
	A13	730	597	743	3413	627
Inhibition	M315	96 ^b	3	3	0	0
	M327	3	97	18	10	0
	M332	8	18	76	0	0
	A13	0	21	0	90	0

^aAnti-anti-idiotypic antisera were incubated with anti-idiotypic monoclonal antibodies on microtiter plates, and then they were tested for the reactivity of ¹²⁵I-labeled anti-idiotypic monoclonal antibodies in sandwich assay. The results are expressed as bound cpm.

^bAnti-anti-idiotypic MoAb M315, M327, M332 and A13 antisera were preincubated with ¹²⁵I-labeled MoAb M315, M327, M332 and A13, and then the mixtures were added to microtiter plates coated with MoAb P1-356. After being washed the bound radioactivity was counted. The results are expressed as percentage of inhibition, compared with that of preimmune serum as a control in inhibition assay.

MoAb-immunized antisera (Ab3) showed inhibitory activity to the binding of anti-Id MoAb (Ab2) to anti-CEA MoAb P1-356 (Ab1).

To investigate the antibodies "Ab1-like Ab3," which react with CEA, the reactivities of four anti-anti-idiotypic antisera to CEA were tested by competition assay, utilizing anti-CEA MoAb P1-356.

As shown in Fig. 2, anti-anti-Id MoAb M315 antiserum, and anti-anti-Id MoAb A13 antiserum competed with ¹²⁵I-labeled MoAb P1-356 to CEA, while anti-anti-Id MoAb M327 antiserum and anti-anti-Id MoAb M332 antiserum did not. This result suggests that anti-anti-Id MoAb M315 and A13 antiserum (Ab3) may have an antibody population of the same specificity as anti-CEA MoAb P1-356 (Ab1). Furthermore, anti-anti-Id MoAb M315 antisera reacted more strongly with CEA than did other antisera and normal sera, as shown in Fig. 3. These results suggest that anti-anti-Id MoAb (Ab3) elicited with anti-Id MoAb M315 could be useful for analysis of the idotype network system.

Development of Anti-Anti-Id MoAb (Ab3)

Syngeneic anti-anti-Id MoAbs were prepared to anti-Id MoAb M315. Thirteen clones were obtained. Among them, anti-anti-Id MoAb 11B2 (IgG₁, Ab3) was selected since it completely inhibited the binding of ¹²⁵I-labeled MoAb M315 (Ab2) to MoAb P1-356 (Ab1). Further experiments were then performed to ascertain whether MoAb 11B2 is an "Ab1-like Ab3 or not." The results of the reactivity to CEA and the competition assay are shown in Table 5. Anti-anti-Id MoAb 11B2 (Ab3) had stronger reactivity to CEA than another anti-anti-Id MoAb 601 (Ab3) (which was elicited with anti-Id MoAb M327); furthermore, the MoAb 11B2 blocked the binding

of ¹²⁵I-labeled MoAb P1-356 to CEA in the competition assay. Table 5 also shows that ¹²⁵I-labeled anti-anti-Id MoAb 11B2 reacted with synthetic CEA peptide P1 and purified CEA, but not with P3, P6, or BSA. ¹²⁵I-labeled anti-anti-Id MoAb 601 did not react with P1, P3, P6, or CEA. These results suggest that anti-anti-Id MoAb 11B2 played a possible role in "Ab1-like Ab3."

Immunohistochemical Study

To investigate the reactivity of anti-anti-Id MoAb 11B2 to CEA, immunostaining in cultured cells and carcinoma tissues was carried out. Anti-anti-Id MoAb 11B2 showed positive staining for CEA-producing cultured cells MKN45, JRST, and WiDr, whereas it showed negative staining for the CEA-non-producing cell RPMI 8226. Irrelevant anti-anti-Id MoAb 601 reacted with none of them, and the staining pattern was similar to that of anti-CEA MoAb P1-356 (Ab1). Representative data are shown in Fig. 4. Immunohistochemical staining in colonic carcinoma tissues was done. As shown in Fig. 5, MoAb 11B2 (Ab3) exhibited a distribution in cancerous tissues similar to that of anti-CEA MoAb P1-356 (Ab1), showing apical labeling of the adenocarcinoma glands. They did not show positive staining for the noncancerous colonic tissue, except for the surface epithelial cells of the colon, with which they faintly reacted.

DISCUSSION

Administration of anti-Id antibodies may trigger an antitumor immune response in hosts under the regulation of an Id-anti-Id interaction, because some of them act as antigen-specific immunomodulators (3,14,15). Therefore, it is important to investigate the structural

Fig. 2. Competition of ^{125}I -MoAb P1-356 binding to CEA by anti-anti-Id antiserum (Ab3). The competitive binding ability of ^{125}I -MoAb P1-356 to CEA coated on microtiter plates after preincubation with anti-anti-Id antisera was compared to that after preincubation with non-immunized mouse serum. anti-A13 (○); anti-M315 (●); anti-M332 (△); anti-M327 (▲).

correlation of idiotopes with antigen and the idiotype network system. On the other hand, CEA is one of the most frequently used tumor markers in clinical medicine and is known to be one of the target molecules on the surface of cancer cells in the immune system. Furthermore, the results of molecular cloning have clarified a conformational and structural change in the CEA molecule (16). We have established monoclonal antibodies specific to the synthetic peptide of CEA (5). We utilized MoAb P1-356 specific to the synthetic peptide (119-140 amino acids) in domain I of CEA as an immunogen for preparation of anti-Id MoAbs. We prepared the anti-Id MoAbs (Ab2) and then the anti-anti-Id MoAbs (Ab3). Seven anti-Id MoAbs were established in a syngeneic system (Table 1). The results shown in Table 2 indicate that all anti-Id MoAbs

selected in this study specifically recognize private idiotopes at the combining site of MoAb P1-356. A cross blocking assay to analyze the spatial relationship among idiotopes on P1-356 showed that there were at least four distinct idiotopes at the combining site (Table 3; Fig. 1) (12). As shown in Table 4, four different anti-Id MoAbs (Ab2) induced anti-anti-Id antibodies (Ab3) that specifically reacted with the corresponding immunizing anti-Id MoAb. The anti-anti-Id antibodies that were induced only with anti-Id MoAb M315 and MoAb A13 were found to have the same reactivity to antigen (CEA) as that of anti-CEA MoAb, whereas the anti-anti-Id antibodies induced with other anti-Id MoAbs (M322 and M327) did not (Figs. 2 and 3). This may indicate that anti-Id MoAbs M315 and A13 bear the internal image of antigen CEA. In order to get the

Fig. 3. Reactivity of anti-anti-idiotypic antiserum to CEA. Each diluted mouse serum was incubated on a coated microtiter plate. After the plate was washed, ^{125}I -labeled goat anti-mouse IgG-Fc antibody was reacted. The plate was washed again and the bound radioactivity was counted in a γ -counter.

TABLE 5. Reactivity of anti-anti-Id MoAb 11B2 to CEA antigen and synthetic peptide

Assay	MoAb			Control
	11B2 (AB3)	601 (AB3)	P1-356 (Ab1)	
Reactivity to CEA ^a	6906 $\pm$ 562cpm ^d	917 $\pm$ 103	23338 $\pm$ 952	906 $\pm$ 112
Competition of ^{125}I -P1-356 binding to CEA ^b	55 $\pm$ 4% ^e	0 $\pm$ 1	87 $\pm$ 5	0 $\pm$ 1
Reactivity of ^{125}I -MoAb to CEA and synthetic peptides ^c				
CEA	5764 $\pm$ 521cpm ^d	2041 $\pm$ 222	18271 $\pm$ 1522	
Synthetic P ₁	5755 $\pm$ 510	2468 $\pm$ 243	13153 $\pm$ 1036	
Peptide P ₃	4128 $\pm$ 382	3026 $\pm$ 298	3438 $\pm$ 324	
P ₆	3611 $\pm$ 358	3411 $\pm$ 348	3797 $\pm$ 366	
BSA	666 $\pm$ 48	533 $\pm$ 36	570 $\pm$ 39	

^aThe supernatants of Ab1 or Ab3 were incubated for 3hrs with CEA coated microtiter plates. After being washed, ^{125}I -labelled anti-IgG. Fc-antibody was added to each well and bound radioactivity was counted in a gamma counter.

^bMicrotiter plates coated with CEA were incubated with an excess of cold anti-anti-idiotypic MoAb (Ab3) and then tested for the ability to bind ^{125}I -labelled MoAb P1-356 (Ab1).

^c ^{125}I -labelled MoAb (Ab3 or Ab1) was incubated with CEA or synthetic peptide coated microtiter plates. Then the plates were washed and bound radioactivity was counted in a gamma counter.

^dResults are expressed as cpm (value denote mean $\pm$ SD).

^eResults are expressed as percentage of inhibition.

anti-anti-Id MoAb (Ab3) specific to CEA, syngeneic anti-anti-Id MoAbs were developed against internal image-bearing anti-Id MoAb M315. As shown in Table 5, the anti-anti-Id MoAb 11B2 selected in this study

showed reactivity to CEA antigen and synthetic peptide P1, while other anti-anti-Id MoAbs, such as MoAb 601, did not. Furthermore, anti-anti-Id MoAb 11B2 (Ab3) competed with ^{125}I -labeled anti-CEA MoAb P1-356

Fig. 4. Immunostaining in cultured cells (CEA-positive MKN45 cells and CEA-negative RPMI 8226) by anti-CEA MoAb P1-356 (Ab1) and anti-anti-Id MoAb 11B2 (Ab3).

(Ab1) to CEA. Immunostaining studies with cultured cancer cells and colonic cancerous tissues showed that anti-anti-Id MoAb 11B2 (Ab3) and anti-CEA MoAb P1-356 (Ab1) had the same reactivities to cultured cells and the same distribution pattern in cancerous tissues, as shown in Figs. 4 and 5. These results suggest that anti-anti-Id MoAb 11B2 (Ab3) has a similar reactivity to anti-CEA MoAb P1-356 (Ab1). This indicates that anti-anti-Id MoAb 11B2 is an "Ab1-like Ab3"-type MoAb, and that anti-Id MoAb M315 is an Ab2 β -type MoAb that bears an internal image of the epitope on CEA. We previously reported (3) that active immunotherapy with Ab2 β is more useful than passive immunotherapy using Ab1 because of the persistent activity. However, previous results showed only that there existed anti-anti-Id polyclonal antibodies (Ab3), induced with Ab2 β and specific to antigen. This study clarified that anti-anti-Id MoAb 11B2 ("Ab1-like Ab3") reacted to CEA antigens, not only in the experiments with purified CEA but also in the immunostaining study with CEA-producing cultured cells and colonic cancerous tissues. Mittelman et al. (4) reported the advantages of internal image-bearing anti-Id antibodies in active

specific immunotherapy of melanoma patients. They showed that treatment with anti-Id MoAb could induce anti-anti-Id antibodies to Ab2 in about 60% of patients with malignant melanoma, resulting in excellent prolongation of survival, and tumor regression in some patients. Patients who had a high level of anti-anti-Id Abs had a better prognosis. These results suggest that the idiotype cascade may be accelerated by Ab2 administration, and that antigen-specific Ab3 may be a beneficial immunological response for cancer patients (15). We previously reported (17) the structural analysis of internal image and comparison of AA sequences between the anti-Id antibody V region and Ag (CEA). In the present study, Ab1 (anti-CEA peptide MoAb), Ab2 β (anti-Id MoAb), and Ab3 (anti-anti-Id MoAb, "Ab1-like Ab3") were established, starting from a definite synthetic peptide (20AA) of CEA as the antigen. It is of interest to analyze the primary structure of Ag, Ab1, Ab2, and Ab3 in the idiotype network system, because such studies may help clarify whether Ab2 functionally mimics Ag, or Ab3 has a reactivity like that of Ab1 (18,19). This study is now under way in our laboratory.

Fig. 5. Staining pattern of colonic cancerous tissues by anti-CEA MoAb (Ab1) and anti-anti-Id MoAb (Ab3) assessed by the immunoperoxidase method. (A) Anti-CEA MoAb P1-356 (Ab1) and (B) anti-anti-Id MoAb 11B2 (1) and (2) denote two different colonic cancer tissues obtained from two patients.

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