

Functional Mimicry of an Anti-idiotypic Antibody to Nominal Antigen on Cellular Response

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One concept for immune therapy of cancer involves induction of antigen mimic antibodies to trigger the immune system into a response against the tumor cells. Anti-idiotypic antibodies (Ab2) directed against the antigen-combining site of other antibodies (Ab1) may functionally and even structurally mimic antigen and induce anti-anti-idiotypic immune response. We report here the generation of murine monoclonal antibody (mAb) WJ02 (Ab2) raised against the murine monoclonal immunoglobulin MJ01 (Ab1), which defines ovarian cancer antigen CA125. In enzyme immunoassays the binding of Ab2 to the variable region of Ab1 could be inhibited by CA125. In addition, the mimicry of mAb WJ02 to CA125 on cellular immune response was detected by human peripheral blood cells. The T cells primed by mAb WJ02 or CA125 proliferated in the presence of CA125 or mAb WJ02, respectively. Furthermore, T cells specific to mAb WJ02 could lyse ovarian cancer cells OVCAR-3 that express CA125. Finally, we proved that a patient immunized with mAb MJ01 could induce T cells that recognize mAb WJ02. In summary, we conclude that mAb WJ02 mimics CA125 on cellular response and such functional mimicry is one of the most important criteria to select Ab2 for cancer therapy.

Key words: Anti-idiotypic antibody — Ovarian cancer — Cellular immunity

Induction of tumor-specific immunity with nominal tumor antigens has achieved limited success when the nominal antigens are not readily available or when the host is tolerant to the nominal antigens. Therefore, an alternative approach to induce internal tumor image antigens is of importance for cancer immunotherapy.

The induction of anti-idiotypic antibodies (anti-Id) Ab2 β as a surrogate antigen for cancer vaccine represents one of the most promising approaches for active immunotherapy.^{1,2} In clinical studies on Ab2 in the human tumor system, Baum *et al.* postulated a beneficial role of Ab2 induced in cancer patients during immunotherapy with anti-CA125 antibody directed against human ovarian carcinoma. They showed that patients who were clinically improved had developed high titers of Ab2 that were maintained for prolonged periods of time.³

The conventional method to detect anti-Id relies on *in vitro* binding and inhibition assays.^{4–6} In these assays it is difficult to distinguish the internal image anti-Id from anti-Id which bind to the idiotopes near the paratope of Ab1 (Ab2 γ). Since genetic restrictions on host immune responses to Ab2 γ may exist, these selecting methods may limit the therapeutic usefulness of Ab2. Although subsequent induction of Ab3 has been demonstrated to correlate favorably with the clinical outcome of Ab2-exposed cancer patients,^{7–9} tumor cell destruction is more likely to be mediated mainly by T cells than by antibodies.¹⁰ Thus, the

correlation between Ab2 and T cells can be used as a parameter to evaluate the anti-tumor effects of Ab2.

Clinical trials of active immunotherapy with Ab1-based vaccines in cancer patients have demonstrated the induction of Ab2.¹¹ However, fewer studies have addressed the issue of stimulating cellular anti-tumor immune responses (T3). Although the induction of epitope-reactive T cells after immunotherapy with murine monoclonal antibody (mAb) has been reported,^{12,13} these epitope-reactive T cells derived from patients were stimulated *in vitro* by original antigens. Therefore, the possibility that these epitope-reactive T cells might be T1 could not be ruled out.

Hence, whether Ab2 can induce antigen-specific T-cell immunity needs to be elucidated. A monoclonal Ab2 (mAb WJ02), induced by immunization of mice with mAb MJ01, a murine mAb specifically recognizing the ovarian cancer antigen CA125, was selected to assess antigen specific T cell response in the current study. We believe that the T cell response observed against CA125-positive cancer cells was based on the recognition of processed mAb WJ02 that has homology to the CA125 sequence.¹⁴ Furthermore, mAb WJ02 was used to detect the T cell response in ovarian cancer patients treated with mAb MJ01 (Ab1).

MATERIALS AND METHODS

Cells The CA125-positive cell lines OVCAR-3 and Caov-4 are human ovarian cancer cell lines obtained from

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American Type Culture Collection (ATCC, Rockville, MD). SK-OV-3 (ATCC) is a CA125-negative ovarian adenocarcinoma cell line. All the cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS).

Generation of anti-Id Murine mAb MJ01¹⁵ conjugated with keyhole limpet hemocyanin (KLH) was used to immunize syngeneic BALB/c mice for the production of anti-Id. Immunization of BALB/c mice, hybridoma fusion as well as cloning and production of ascites in large quantities from mice were done as described elsewhere.¹⁶ Selection of anti-Id (Ab2) was performed by a direct binding assay as described below. The Ab2, anti-Id WJ02 (IgG1), was purified from ascites by affinity chromatography on a protein G column. The purity of the isolated immunoglobulin (>98%) was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Direct binding of mAb WJ02 to scFv-MJ01 The ability to recognize idiotopes on Ab1 by mAb WJ02 was detected by ELISA. A single chain antibody, scFv-MJ01, developed by the method described previously¹⁷ was selected for this assay. Briefly, Maxisorp Lock plates (Nunc, Roskilde, Denmark) were coated with 100 μ l of scFv-MJ01 (1 μ g/ml) overnight at 4°C. Then the plates were blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature and incubated with mAb WJ02 (41 ng/ml). After 1-h incubation, the plates were washed with 0.1% Tween 20/phosphate-buffered saline (PBS), then peroxidase-labeled goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) was added, and the plates were incubated for another hour. Then the plates were washed and filled with 2,2'-azino-bis-(3-ethylbenzthiazolinesulfonate) (ABTS) substrate. The absorbance was read at 405 nm. A non-related mouse IgG1 antibody MOPC-21 (Sigma, St. Louis, MO) was selected as a negative control.

Inhibition assay An inhibition assay was developed to determine whether CA125 would specifically inhibit binding of mAb WJ02 to the variable region on mAb MJ01, the antibody used to raise mAb WJ02. Briefly, Maxisorp Lock plates were coated with 100 μ l of scFv-MJ01 (1 μ g/ml) overnight at 4°C. The plates were then blocked with 3% BSA/PBS for 1 h at room temperature. Solutions of mAb WJ02 (41 ng/ml) preincubated with CA125 (Southern Biotech) or a non-related antigen CA15.3 (Southern Biotech) at different concentrations (2700, 1350, 675, 42, 10.5, 0 U/ml) were then added to the plates. After the 1-h incubation at room temperature, the plates were washed as previously described. Then peroxidase-labeled goat anti-mouse IgG1 was added (100 μ l/well). ABTS substrate was added after 1-h incubation at room temperature. The absorbance was read at 405 nm.

T cell proliferation assay

CA125 specific T cells in response to mAb WJ02: The method used to select antigen-specific T cells was that

described by Barnd *et al.*¹⁸ Briefly, human peripheral blood cells (PBMC) were obtained from two healthy donors and layered on a Ficoll density gradient. After 30 min centrifugation at 1500g, the interface containing lymphocytes were collected and washed three times in AIM medium. The cells were plated at 10⁵ cells per ml in AIM medium. Irradiated (6000 rad) Caov-4 cells or SK-OV-3 cells (10⁴/ml) were added as a stimulation antigen at the initiation of the culture. After up to 4 weeks of incubation in a humidified CO₂ incubator, cells were pulsed in triplicate with either mAb WJ02 or MOPC-21 at 0, 5, or 10 μ g/ml. In 3 days, proliferative lymphocyte response was determined in a standard [³H]thymidine incorporation assay. Stimulation index (SI) was calculated as follows: Stimulation index=(cpm in PBMCs with stimulant/cpm in PBMCs without stimulant).

Proliferation of T cells specific to mAb WJ02 in response to CA125-positive cells: PBMCs were isolated by Ficoll as described above. After having been stimulated by mAb WJ02 or IgG1 (10 μ g/ml) for 4 weeks in a humidified CO₂ incubator, the cells were washed once with AIM medium. Then irradiated (6000 rad) OVCAR-3 or SK-OV-3 cells were added in AIM medium, and the cells were incubated in a humidified incubator for 3 days. Proliferative responses were determined by [³H]thymidine incorporation assay.

Using Ab2 to detect T3 response induced by Ab1 *in vivo* Recently, we have completed an active immunization protocol in patients who had their primary tumor removed before immunotherapy. Patients with FIGO stage III–IV ovarian cancer were repeatedly immunized with mAb MJ01. Induction of Ab2 specific cellular immunity was analyzed *in vitro* by means of a proliferation assay. Briefly, PBMCs from patients before and after treatment were washed twice with AIM medium and 5 \times 10⁵ cells per well were incubated with CA125 (1000 U/ml), mAb WJ02 (0.1 μ g/ml), MOPC-21 (0.1 μ g/ml), in AIM medium. The nonspecific mitogen phytohemagglutinin-P (Sigma) was used as a positive control at 10 μ g/ml. After having been incubated for 3 days in an atmosphere containing 5% CO₂, the cells were pulsed with [³H]thymidine. [³H]Thymidine incorporation was determined in terms of the stimulation index.

Cytotoxicity assay The cytotoxic activity of the T cells was determined in a 6-h ⁵¹Cr-release assay. OVCAR-3 or SK-OV-3 cells growing in monolayer in tissue culture flasks were digested with a trypsin/EDTA dispersal solution and incubated with ⁵¹Cr for 1 h at 37°C. These target cells were washed three times in AIM medium and then seeded into microtiter plates. Effector T cells stimulated by mAb WJ02 (10 μ g/ml) for 4 days were washed with AIM medium and added to ⁵¹Cr-labeled target cells (E:T=10:1). The plates were incubated for 6 h at 37°C in a humidified atmosphere of 5% CO₂. After this incubation

period, supernatant fluid was harvested and counted in a γ counter (LKB, Turku, Finland). All tests were carried out in triplicate. The percentage of specific ^{51}Cr release was calculated according to the following formula: % specific lysis = (sample release cpm - spontaneous release cpm) / (maximum release cpm - spontaneous release cpm) \times 100. Triton was used to obtain maximum release.

RESULTS

Anti-idiotypic specificity of mAb WJ02 The anti-idiotypic binding properties of mAb WJ02 were first evaluated in a direct binding assay (Fig. 1). A single chain MJ01 that is composed of V_L and V_H of mAb MJ01 was employed in this study to avoid nonspecific binding. The result showed that mAb WJ02 reacted with scFv-MJ01, which indicates that mAb WJ02 recognizes the determinant presented on the variable region of mAb MJ01, and the reaction did not occur to isotype control antibody. The specificity of binding to the variable region of Ab1 was further confirmed by an inhibition assay (Fig. 2). When coincubated with antigen CA125, the binding of anti-Id WJ02 to scFv-MJ01 was greatly inhibited. At the concentration of 2700 U/ml, the antigen could inhibit 50% of the binding of mAb WJ02 to scFv-MJ01. This inhibition was CA125-specific, because no inhibition was observed when CA15.3 was used. These results indicate that mAb WJ02 recognizes an idiotypic within the antigen-combining site of mAb MJ01.

CA125-specific T cell responses to mAb WJ02 CA125 specific T cells were obtained by incubating the PBMCs from two healthy donors with irradiated CA125-positive tumor cells. The ability of these T cells to incorporate [^3H]thymidine in the presence of mAb WJ02 was monitored. PBMCs stimulated by irradiated Caov-4 cells showed a significant proliferative response after *in vitro* stimulation with mAb WJ02. This response was specific to Ab2, since no proliferation was observed when cells were stimulated by control IgG1 (Fig. 3a). In addition, T cells selected by CA125-negative tumor cells did not proliferate in the presence of mAb WJ02 (Fig. 3b). These results indicated that mAb WJ02 could induce a secondary proliferation of T cells stimulated by CA125. Anti-Id dose dependency of proliferative PBMC response is shown in Fig. 3a.

Proliferation of mAb WJ02 primed T cells to tumor cells To investigate induction of cellular anti-tumor responses to mAb WJ02 in the human system, proliferative responses of T cells from two donors were measured. Cells were primed with mAb WJ02 and secondarily challenged with either OVCAR-3 or SK-OV-3 cells. T cells produced a significantly increased secondary proliferative response *in vitro* when challenged with irradiated CA125-positive cells, while no response was observed when they were challenged with irradiated CA125-negative cells. Fig.

4 shows that mAb WJ02 would prime human T cells to make a proliferation response to cells bearing CA125. The IgG1 priming has no effect on the response against the same cell line. The significance of these results is that T cells primed and stimulated by mAb WJ02 are cross-reactive with CA125, and therefore from a vaccination point of view such cells will recognize CA125 upon encountering the tumor cells.

Evaluation of the T cell responses elicited by mAb MJ01 in cancer patients The development of cellular immunity induced by immunization with mAb MJ01 was

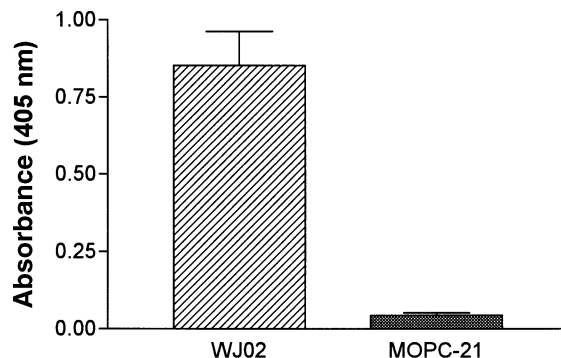


Fig. 1. Binding activity of mAb WJ02 to Ab1. Microtiter plates coated with scFv-MJ01 (1 $\mu\text{g}/\text{ml}$) were incubated with mAb WJ02 (41 ng/ml). After washing, peroxidase-labeled goat anti-mouse IgG1 was added to detect the binding of mAb WJ02. Data are mean values of triplicate readings (405 nm) \pm SD.

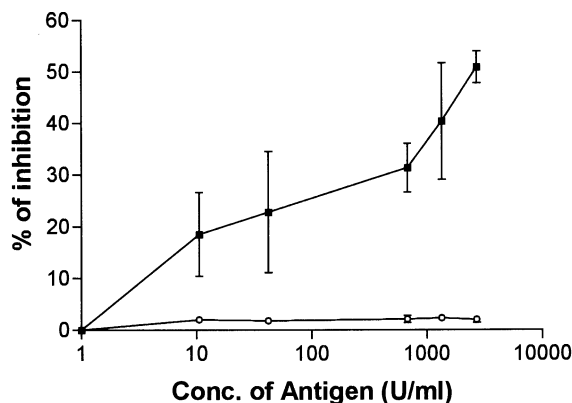


Fig. 2. Binding inhibition of mAb WJ02 to Ab1 by CA125. Microtiter plates coated with scFv-MJ01 (1 $\mu\text{g}/\text{ml}$) were incubated with a mixture of mAb WJ02 (41 ng/ml) and various concentrations of CA125 or CA15.3 (2700, 1350, 675, 42, 10.5, 0 U/ml). After washing, peroxidase labeled goat anti-mouse IgG1 was added to test for bound mAb WJ02. Results are expressed as percentages of inhibition. Data are mean values of triplicate readings \pm SD. ■ CA125, ○ CA15.3.

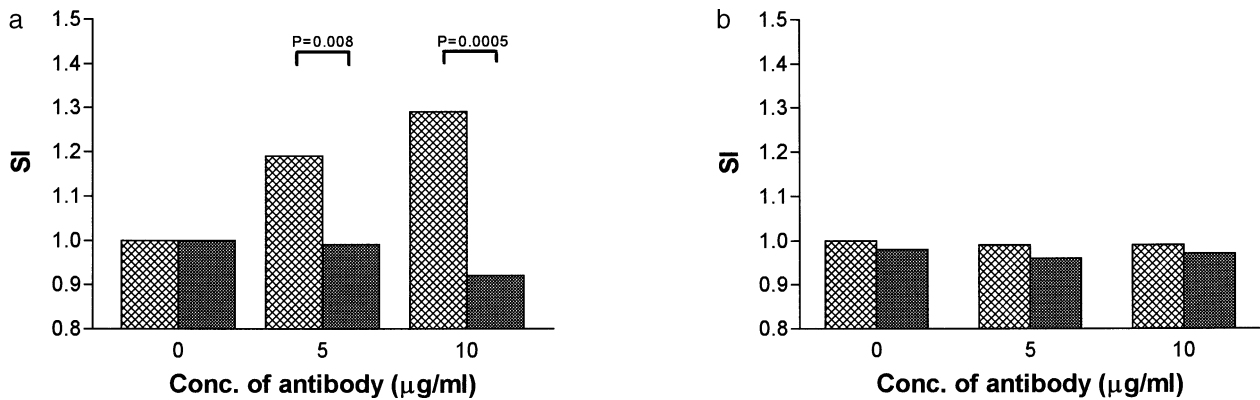


Fig. 3. CA125-primed T-cell proliferation (expressed as SI) in the presence of mAb WJ02. PBMCs were isolated from blood of two healthy donors and cultured with irradiated CA125-positive (Fig. 3a) or CA125-negative (Fig. 3b) cells for 4 weeks. The cells were then challenged with mAb WJ02 at 0, 5, or 10 µg/ml. [³H]Thymidine incorporation was measured in 3 days. Data are mean values of two experiments performed in triplicate. *P* values were calculated by paired *T* test using PRISM (version 3.0). ▨ WJ02, ■ MOPC-21.

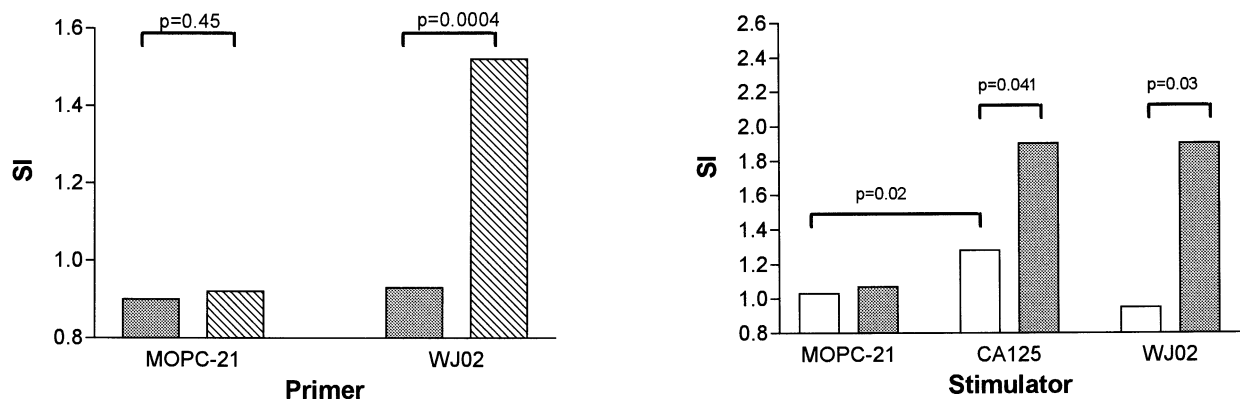


Fig. 4. The specific response of anti-Id WJ02 specific T cells to CA125-positive tumor cells. Ab2 specific T cells were obtained by incubating the PBMCs from two healthy donors with mAb WJ02 (10 µg/ml) for 4 weeks. Control T cells were obtained by incubating PBMCs with an isotype-matched antibody (MOPC-21). The proliferation of T cells in response to irradiated CA125-positive (OVCAR-3) or CA125-negative (SK-OV-3) tumor cells was monitored in terms of [³H]thymidine incorporation. Data are mean values of two experiments performed in triplicate. *P* values were calculated by paired *T* test using PRISM (version 3.0). ■ SK-OV-3, ▨ OVCAR-3.

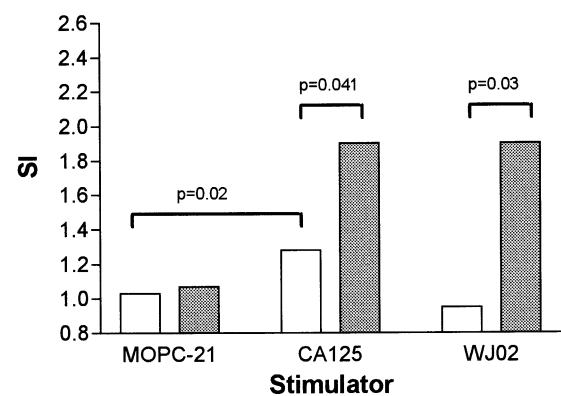


Fig. 5. T cell proliferation (expressed as SI) in the presence of mAb WJ02 and CA125 in one patient treated with mAb MJ01. PBMC proliferative responses of the patient either before therapy or after the last immunization were stimulated with the optimal concentration of mAb WJ02 (0.1 µg/ml) or CA125 (1000 U/ml). Proliferative responses were determined 3 days later in a [³H]thymidine incorporation assay. MOPC-21 was used as isotype control for mAb WJ02. *P* values were calculated by paired *T* test using PRISM (version 3.0). □ pretreatment, ▨ posttreatment.

assessed by testing PBMCs obtained from patients before and after treatment with the vaccine. T cell response was measured in terms of the proliferation of PBMCs incubated with CA125, mAb WJ02 and isotype-matched control antibody MOPC-21, respectively. Positive proliferative responses were found in two of four patients. Representative data from one patient are illustrated in Fig. 5. After treatment with mAb MJ01, PBMCs from this patient

showed a significant and specific proliferative response after *in vitro* stimulation with either mAb WJ02 or CA125 as compared with stimulation of control IgG1. The slight proliferative response to CA125 before treatment indicates the existence of T1.

Tumor lysis by cytotoxic T lymphocyte (CTL) primed with mAb WJ02 As mentioned by Wahab and Metzgar, a specific CTL response against pancreatic tumor cells can be generated from lymph node cells due to *in vitro* stimu-

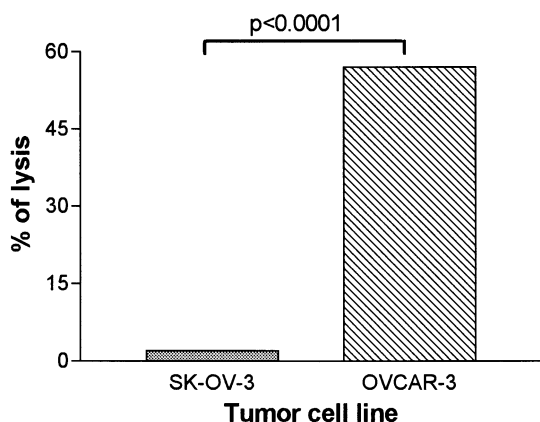


Fig. 6. Cytolytic activity of mAb WJ02 primed T cells on CA125-positive tumor cells (OVCAR-3) and CA125-negative tumor cells (SK-OV-3) at effector to target ratio of 10:1. The *P* value was calculated by paired *T* test using PRISM (version 3.0).

lation with allogeneic pancreatic tumor cells.¹⁹⁾ In order to determine whether T cells selected by mAb WJ02 have the ability to lyse antigen-expressing tumor cells, we performed a 6-h ⁵¹Cr release assay. As shown in Fig. 6, CA125-positive tumor cells (OVCAR-3) were lysed by mAb WJ02-primed T cells, while CA125-negative tumor targets (SK-OV-3) were only weakly affected. This result implied that mAb WJ02 could induce a specific CTL response to CA125-positive tumor cells and such ability of Ab2 could be determined in an allogeneic system.

DISCUSSION

A major challenge in cancer therapy is to trigger an active immune response against tumor antigens which can “tolerize” cancer patients. Ab2 β bearing internal images of human tumor-associated antigen is a potential modulator of the immune response in malignant diseases. Presentation of anti-idiotypes as surrogate antigen might help the host overcome the tolerance to malignant cells. It is well established in several systems that monoclonal anti-Id can potentially play a role in vaccine development, by virtue of their ability to mimic the nominal antigen and to stimulate the immune system.^{20, 21)}

Since the studies in murine tumor systems have clearly shown that Ab2 can induce specific and protective immunity, a number of anti-Ids have been generated in goats, mice and rabbits that mimic the original antigens.²²⁾ The traditional criteria for the determination of Ab2 are to compete with antigens *in vitro* and to induce antigen-specific antibodies *in vivo*.⁵⁾ For instance, the anti-Id WJ02 used in this study could be verified to contain the internal image of CA125 epitope because of its ability to bind to the antigen-combining region of mAb MJ01, to inhibit the

binding of CA125 to scFv-MJ01, and to induce CA125-specific antibody (Ab3) in rats (data not shown). However, cellular response is more crucial than humoral response in antitumor therapy. In this regard, the ability of anti-Id to invoke T-cell-mediated anti-tumor responses may be more relevant to effective therapy than its ability to induce a humoral anti-tumor response. Thus, induction of protective cellular response should be included in the criteria to select anti-Id for cancer therapy. Developing a method to predict which antibody will be effective on cellular response in patients will greatly improve the anti-Id approach in tumor immunotherapy.

Since the antigen mimicry properties of Ab2 β are species-independent, mouse Ab2 β is potentially suitable as a surrogate antigen in human cancer patients. This is why we generated a murine anti-Id to assess its influence on cellular immunity in human. Our results showed that when the human PBMCs were exposed to mAb WJ02, the protein was likely to be internalized, degraded to peptides and presented to T cells by the antigen-presenting cells. When these stimulated T cells were further challenged by antigen-expressing tumor cells, they responded positively by proliferation. For mAb WJ02-primed T cells to recognize CA125-positive tumor cells, it is necessary that the amino acid sequence of mAb WJ02 has linear homology to that of CA125. To identify the epitopes responsible for the mimicry between Ag and anti-Id, an extensive structure and sequence analysis should be performed.^{23, 24)} Unfortunately, the sequence and three-dimensional structure of CA125 have not yet been identified.

A major criticism regarding antibody therapy is that the induction of a humoral anti-tumor response through the idiotypic cascade may not be sufficient for tumor rejection. However, no appropriate animal model systems exist, which can be used to predict T cell cytotoxic functions in humans. Moreover, Ab2 induced cytolytic T cells, which are reactive to human tumors, can not be investigated in experimental animal systems when transfected tumor cells are not available. This is because cytolytic reactivities of T cells are generally restricted to syngeneic targets.²⁵⁾ In this communication we selected an *in vitro* allogeneic system to detect the anti-tumor effect of the T cells primed by mAb WJ02. Anti-Id specific T cells specifically lysed CA125-positive tumor cells, but had no effect on CA125-negative tumor cells. This indicates that mAb WJ02 is able to prime T cells to elicit specific anti-tumor responses *in vitro* and suggests that the responses observed in the allogeneic system may be directly relevant to likely responses in cancer patients. However, the precise target specificities and phenotypes of the induced T cells remain to be determined.

The mimicry of mAb WJ02 to CA125 on cellular response was also confirmed by other two experiments. PBMCs selected by CA125-positive cells proliferated in

response to mAb WJ02. Furthermore, PBMCs derived from patients treated with mAb MJ01 had proliferative responses to both CA125 and mAb WJ02. Recently, murine Ab1 mAb MJ01 has been used in active immunization protocols in human ovarian cancer patients. Therefore, a better understanding of the effective mechanisms in patients is needed. The patient selected for this study had a T cell response initiated by both Ab2 and CA125. This fact implies that these T cells are T3, because mAb WJ02 did not invoke T1 in pretreatment samples. Therefore, mAb WJ02 could be used to detect T3 response induced by mAb MJ01.

T cell response induced in patients in different clinical protocols showed that Ab2 presented a stronger anti-tumor effect than Ab1.^{13,26,27} Therefore, multiclonal Ab2 β are more suitable to carry diversified “internal images” of the antigen. The balance of advantages for multiclonal Ab2 vs. Ab1 remains to be determined, but it is likely that the

induction of Ab2, which can be effectively presented to T cells, would have the greater therapeutic value.

On the basis of the present findings, we believe that besides humoral response, cellular response is another important criterion to select Ab2 for cancer therapy. These internal images are probably a small fraction of all anti-Id, but once determined, they are exceptional candidates for the development of therapeutic agents for cancer.

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