Monoclonal Antidiotypic Antibodies Related to the p97 Human Melanoma Antigen

Maria Kahn, Ingegerd Hellström, Charles D. Estin, and Karl Erik Hellström
ONCOGEN, Seattle, Washington 98121

ABSTRACT

We have made monoclonal antidiotypic antibodies (Ab2) relating to the p97 antigen of human melanoma. This was accomplished by immunizing BALB/c mice with 96.5, a monoclonal antibody (Mab) specific for epitope p97, hybridizing their spleen cells with NS-1 myeloma cells, and selecting for hybridomas making antibody binding to Fab fragments prepared from Mab 96.5 (Fab 96.5). The Ab2 were tested for binding to Fab 96.5, as well as for their ability to inhibit the binding between Mab 96.5 and p97. Three monoclonal Ab2 were identified which competitively inhibited the binding between p97 and Mab 96.5 when injected into either BALB/c or C3H/HeN mice; two of them induced Ab3 which expressed the same idiotype as Mab 96.5 and which were specific for p97. These two Ab2 thus appear to functionally mimic p97. They were, however, unable to induce delayed-type hypersensitivity to p97 and to protect mice against transplants of p97-positive mouse melanoma cells, suggesting that the epitope recognized by Mab 96.5 may not be a target for cell-mediated rejection of tumors.

INTRODUCTION

Antidiody antibodies offer promise as "vaccines" to various infectious agents (1-4), and they might be used to induce immune responses to tumor antigens as well (5-9). For example, Kennedy et al. (10) showed that mice transplanted with SV40-induced tumors had enhanced survival if first immunized with antidiody antibodies to SV40. Dunn et al. (11) reported that monoclonal antidiody antibodies protected rats against challenge with a syngeneic sarcoma. Raychaudhuri et al. (12) used monoclonal antidiody antibodies to induce a cytolytic T-cell response in mice, which protected against tumor challenge. Koprowski et al. (13) observed occasional regressions of gastrointestinal carcinoma after patients received an antitumor-like Mab 8.2, could immunoprecipitate p97. In vivo therapy experiments utilized 2BF/1, also from K-1735-M2, which expresses approximately $5 \times 10^7$ p97 molecules/cell (15). In vitro, and two of them induced antibodies to p97 molecules/cell subsequent to transfection with the gene for p97. The K-1735-M2 cells, which entirely lack p97, are referred to as par cells, since the 2a and 2BF/1 lines were derived from them.

Antidiody antibodies. Hybridoma 96.5, which produces an IgG2a Mab to epitope p97" of the human melanoma-associated antigen p97, was obtained by fusing spleen cells from an immunized BALB/c mouse with NS-1 myeloma cells (19). Fab fragments were made from Mab 96.5 by papain digestion and are referred to as Fab 96.5 (19). Mab F6 is an IgG2a specific for a proteoglycan antigen on human melanoma cells (20). It is used to prepare Fab fragments (referred to as Fab F6), which were employed as controls. Mab MPG24 is an IgG2b specific for the melanoma-associated proteoglycan (20), L6 is an IgG2a to a carbohydrate antigen expressed on most human carcinomas (21), and P1.17 is an IgG2a myeloma protein (American Type Culture Collection, Rockville, MD).

In order to raise Ab2, BALB/c mice were treated s.c. with 100 $\mu$g of purified Mab 96.5 which had been conjugated with KLH and subsequently mixed with Freund's complete adjuvant (Bacto H37R; Difco Laboratories, Detroit, MI). One month later, they were treated i.p. with the same amount of KLH-conjugated Mab 96.5 in Freund's incomplete adjuvant (Difco). The mice were subsequently treated with Mab 96.5 in saline at 2-week intervals for 1 or 2 more times. Three days after the last injection, they were killed and a spleen cell suspension was prepared and fused with NS-1 mouse myeloma cells, using standard techniques (21).

Screening of Hybridomas. Primary screening was performed by an ELISA (21). Fab 96.5, at a concentration of 4 $\mu$g/ml in PBS, was plated onto Immulon plates (Dynatech Laboratories, Chantilly, VA). The next day, the plates were washed with PBS containing 0.05% Tween 20 and "blocked" by incubation for 1 h with PBS containing 0.05% Tween and 1% fetal calf serum. Supernatants (50 $\mu$l) were added from each well with growing hybridoma cells. This was followed, 1 h later, by a mixture of goat anti-mouse IgG1 (Zymed, San Francisco, CA), which had been coupled with HRP and to which 0.05% Tween 20 in PBS and 1% fetal calf serum had been added. After 1-h incubation, antibody binding to the plated Fab 96.5 was detected by adding OPD, according to the directions of the manufacturer (Zymed). The plates were read in an
ANTIDIOTYPIC ANTIBODIES TO p97

automatic microplate reader (Genetic Systems Corporation, Seattle, WA) at an absorbance of 492 nm/630 nm. Fab fragments from MAb F6 (20) were employed as controls, and only those hybridomas which made MAb binding to Fab 96.5 but not to Fab F6 were retained for further testing. Note that the screening procedures used only detected hybridomas making IgG1 antibodies.

Hybridomas which made antibodies binding to Fab 96.5 but not to Fab F6 were cloned twice by limiting dilution, after which they were expanded and injected into pristane-primed BALB/c mice for ascites production.

Studies on Purified Antidiotype. To identify Ab2, which could interfere with the binding of MAb 96.5 to p97, SK MEL-28 melanoma cells were plated at 10^4 cells/well. Ab2 purified by precipitation with saturated ammonium sulfate and ion exchange chromatography (22) were mixed with MAB 96.5 (1 µg/ml) or a control MAB (F6). Inhibition of binding of MAB 96.5 to the melanoma cells were detected by addition of a goat anti-mouse HRP conjugate as above; some tests of this type were also performed on hybridoma supernatants.

To study whether the purified Ab2 could block the antigen binding site of Fab 96.5, various concentrations of Ab2 were added to wells of Immulon plates onto which Fab 96.5 had been plated. p97 antigen isolated from transfected mouse melanoma cells (15) was radiolabeled, an amount giving 2 x 10^5 cpm/well was added, and the number of counts bound/well was determined.

Competition of Radiolabeled p97 for Binding to 96.5 Fab. Various concentrations of purified Ab2 were mixed with a constant amount of 125I-labeled p97 antigen, and the mixtures were added to plates which had been coated with Fab 96.5. After 1 h, the plates were washed, 2 N NaOH was added, and the contents of the wells were counted in a gamma counter.

Searches for Ab3 in Vivo. BALB/c and C3H/HeN female mice, 6 to 8 weeks-old, were treated i.p. with 50 µg of Ab2 conjugated to KLH and mixed with complete adjuvant. Five days later, they were given a booster injection of Ab2 in incomplete adjuvant and were subsequently treated with Ab2 in saline at 5-day intervals. After a total of four and six immunizations, respectively, the mice were bled. They continued to be given booster injections at 2-week intervals for several weeks. In some cases, the immunization protocol was initiated at 2-week intervals with four or five boosts.

Serum from the immunized mice were titered for the presence of antibodies binding to Ab2, referred to as Ab3. This was done by mixing diluted sera with Ab2 and adding the mixture to Immulon plates which had been coated with Fab 96.5 (as Ab1), after which goat anti-mouse IgG1-HRP and OPD were added. Data were expressed as percentage inhibition of the binding of Ab2 to Ab1. They were calculated by determining the absorbance value for Ab3 plus Ab2, dividing it by the absorbance value for Ab2 alone, and subtracting the quotient from 100.

The sera were also tested for Ab3 binding to the p97 antigen. Purified p97 (15, 17) was plated onto Immulon plates at 5 µg/ml in PBS and left overnight. After blocking, diluted sera were added, followed by a goat antiserum to mouse immunoglobulin which reacted with IgG, IgM, and IgA and was coupled to HRP.

A solid phase inhibition assay was also employed, in which the mouse sera were mixed with 2α mouse melanoma cells, which express p97 at the cell surface, or with par mouse melanoma cells, which do not. The mixture was first incubated for 1 h and then added to Immulon plates coated with the p97 antigen. As above, binding was detected by adding OPD in the presence of goat anti-mouse HRP conjugates.

DTH. Six-week-old (C3H/HeN) female mice were immunized with v-p97NY, as previously described (15, 23) or by injection of various concentrations of Ab2 No. 3, which was either unconjugated or conjugated with KLH and administered s.c. at 4 four sites. Six days later, 5 mice/group were challenged with 20 µl of PBS containing p97-positive 2α or p97-negative par cells (5 x 10^4 cells/mouse) in the left hind footpad and with 20 µl of PBS alone in the contralateral right hind footpad. Twenty-four h later, the thickness of the footpads was measured in a double blind manner, using a micrometer. The thickness of the PBS-injected footpad was subtracted from that of the experimental footpad, and the incremental swelling was calculated for each mouse.

Assay for Antitumor Activity in Vivo. Female C3H/HeN mice, approximately 6 weeks old, were given i.p. injections of various doses of Ab2 Nos. 3 or 7, 3 times during 21 days; immunization with v-p97NY (or v-NY, as a control) was carried out as previously described (15, 23). The Ab2 were administered either conjugated with KLH or unconjugated and either with or without Freund’s adjuvant. Seven days after the last boost, the mice were challenged i.v. with 5 x 10^7 2BF/1 cells. Lungs were removed, fixed for 24 h in Bouin’s fixative, and washed in water. This allowed whitish metastases to be seen against yellow normal lung tissue. The metastases were enumerated and the data were presented as the means for the total number of metastases/mouse and the SD of the means. All evaluations of the lungs were done “blindly” on coded samples.

RESULTS

Generation of Ab2 Binding to Idiotypic Determinants on MAb 96.5. BALB/c mice were immunized with MAB 96.5, their spleen cells were fused, and hybridoma supernatants were screened for IgG1 antibodies to MAB 96.5, as described in “Materials and Methods.” Approximately 3000 hybridomas were obtained from eight different fusions. Supernatants from 70 of these hybridomas were found to bind to Fab 96.5 and not to the control Fab F6, and most of the hybridomas from which they were derived, therefore, were presumed to make Ab2. Seven of these hybridomas were cloned, and the MAB which they made were purified and tested for binding to Fab 96.5. None of the seven Ab2 bound to P1.17, MPG24, or L6, which were used as controls (Table 1).

Tests on Ab2 Specificity for the Antigen Binding Site of MAB 96.5. Our next step was to investigate whether any of the seven Ab2 appeared to identify the antigen binding site of MAB 96.5. First, we measured their ability to inhibit the binding of MAB 96.5 to the p97 antigen, as expressed by SK MEL-28 cells; this was tested as outlined in “Materials and Methods.” Three of the seven Ab2, Nos. 3, 5, and 7, strongly inhibited this binding, while two Ab2 (Nos. 4 and 6) gave a weak inhibition and two (Nos. 1 and 2) did not inhibit at all (Fig. 1).

Second, we studied the ability of the seven Ab2 to compete with soluble p97 for binding to MAB 96.5. Various dilutions of each Ab2 were mixed with radiiodinated p97 and the binding of p97 to Fab 96.5 was determined in a solid phase assay (Fig. 2). Ab2 Nos. 3, 5, and 7 competed with radiiodinated p97 while the four other antiidiotype antibodies (Nos. 1, 2, 4, 6) did not. The 100% binding value (binding of 125I-labeled p97 to Fab 96.5) was 2500 cpm. The results were thus similar to those presented in Fig. 1.

Third, we could show, as summarized in Fig. 3, that the same three Ab2, namely Nos. 3, 5, and 7, could block the antigen-binding sites of Fab 96.5 so as to decrease 50–60% the binding of radiiodinated p97 to Fab 96.5. One Ab2, No. 2, gave 25% inhibition of this binding and the remaining three Ab2 (Nos. 1, 4, and 6) gave 0–10% inhibition. These data correlate well with

<table>
<thead>
<tr>
<th>Table 1</th>
<th>MAb 96.5 specifically inhibits the binding of seven Ab2 to Fab 96.5 as detected by an ELISA assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>Ab2</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>0.961</td>
</tr>
<tr>
<td>2</td>
<td>1.074</td>
</tr>
<tr>
<td>3</td>
<td>0.655</td>
</tr>
<tr>
<td>4</td>
<td>0.649</td>
</tr>
<tr>
<td>5</td>
<td>0.555</td>
</tr>
<tr>
<td>6</td>
<td>0.447</td>
</tr>
<tr>
<td>7</td>
<td>0.554</td>
</tr>
</tbody>
</table>
Fig. 1. Ab2 can inhibit the binding of MAb 96.5 to SK MEL-28 cells. MAb 96.5 (0.33 μg/ml) was mixed with Ab2 (10 μg/ml) and added to SK MEL-28 cells, which express approximately 400,000 molecules of p97/cell, that had been plated onto the wells of Immulon plates. The binding of MAb 96.5 to the cells was detected by an ELISA assay.

Fig. 2. Competitive inhibition of the binding of 125I-labeled p97 antigen by Ab2.

Fig. 3. Ab2 can inhibit the binding of Fab 96.5 to radiolabeled p97. Fab 96.5 fragments were plated onto the wells of Immulon plates and Ab2 (10 μg/ml) was added. It was tested whether the binding of radiolabeled p97 to the antibody-coated wells was inhibited, as would be the case if the p97-binding sites on Fab 96.5 were blocked.

those presented in Fig. 2, where Ab2 Nos. 3, 5, and 7 gave close to 100% inhibition when tested at a concentration of 10 μg/ml, as compared to less than 40% inhibition for any of the other Ab2. Likewise, soluble p97 antigen could compete with Ab2 for binding to MAb 96.5 (data not shown) when tested as previously described (7).

Taken together the data are compatible with the notion that three Ab2, Nos. 3, 5, and 7, are capable of functionally mimicking the p97* epitope (1, 2). They do, however, not rule out the possibility that steric hinderance was responsible for the effects observed. In order to better study this, future work will utilize, as inhibitors, small peptides encoded by the p97 gene.

Induction of an Ab3 Response. We then investigated whether any of the seven Ab2 could induce an Ab3 antibody response in mice. This was first done in the syngeneic (BALB/c) strain. For most of the experiments, the Ab2 were conjugated with KLH.

After immunization with Ab2, sera of the mice were tested for the presence of Ab3, detectable by its ability to bind to Ab2. Several dilutions of the mouse sera were mixed with the respective Ab2 and added to Immulon plates coated with Fab 96.5 (as Ab1). Goat anti-mouse IgG1-HRP was then used to detect the binding of the Ab2 to the Fab 96.5. After immunization with any of the Ab2 but No. 6, serum derived already from the first bleeding contained antibodies which inhibited 70–100% the binding of Ab2 to Fab 96.5. This inhibition was close to 100% when sera were tested after the second bleeding, and sera from mice given No. 6 were then also strongly inhibitory (data not shown).

As the next step, we tested whether sera from the immunized mice could bind to p97 (like an Ab1). The reason for choosing soluble p97 rather than transfected tumor cells as the target was to reduce the risk of obtaining false positive results. For the same reason, absorption tests rather than binding assays with cells were performed to verify Ab3 specificity. Soluble p97 antigen was plated and diluted mouse sera were added (after blockage), followed by goat anti-mouse IgG-HRP. Sera from mice immunized with either of two Ab2, No. 3 or 7, bound to p97, while sera from mice immunized with any of the other five Ab2, including No. 5, did not (Fig. 4); the difference between the results obtained with sera from mice immunized with either of the two Ab2 referred to as No. 3 or 7 was statistically significant (P < 0.01) from the group in which P1.17 was used as the immunogen. Normal mouse serum (data not shown) did not contain antibodies binding to p97. A comparison was done in which we titrated, as a positive control, either MAb 96.5 or a mouse anti-p97 serum and compared the binding observed with that seen with sera from mice immunized with either of the two Ab2 referred to as No. 3 or 7. The data indicated that the latter sera contained p97-specific Ab3 in a concentration of approximately 1–5 μg/ml.

To further establish the specificity of the Ab3 for p97, sera from Ab2-immunized mice were absorbed with 1 × 10⁶ cells from either the p97-positive mouse melanoma line 2a or from its p97-negative parent (as a control), before they were added to p97 which had been coated onto plates. The binding of the Ab3 to p97 was then detected by using a goat anti-mouse IgG-HRP conjugate. As shown in Fig. 5, A and B, sera from mice immunized with either No. 3 or 7 contained Ab3 which bound to p97, and this Ab3 activity was significantly (P < 0.05) removed by absorption with 2a cells but not by absorption with par cells. Since there was a lower binding to p97 with sera from mice immunized with either Ab2 No. 4 or 5, the absorption data with these sera were less clear (Fig. 5, C and D).
ANTIIDIOTYPIC ANTIBODIES TO p97

Fig. 4. A, binding, to plated p97, of mouse sera containing Ab3. The sera were derived from BALB/c mice which had been immunized with anti-idiotypic antibody-conjugated to KLH. B, binding, to plated p97, of MAb 96.5 and of sera pooled from mice immunized to p97.

Fig. 5. Inhibition of the binding, to plated p97, of Ab3 from BALB/c mice by absorption with p97-positive (2a) cells but not by absorption with p97-negative (par) cells. A, data from mice immunized with Ab2 No. 3; B, C, and D, data after immunization with Ab2 Nos. 7, 4, and 5, respectively.

Fig. 6. A, binding, to plated p97, of purified MAb 96.5 by absorption with p97-positive (2a) cells (A). A similar experiment using, instead, serum from mice immunized with p97 is presented in B. Serum from mice immunized with P1.17 (C) or normal mouse serum (NMS) (D) did not bind to p97.

Fig. 6 shows that the binding of purified MAb 96.5 to p97 was completely abrogated by absorption with 2a cells, while the binding of serum antibodies from mice immunized with p97 was only partially inhibited. This is interesting since the finding with the serum antibodies was analogous to the data obtained with the Ab3 sera from mice immunized with either No. 3 or 7, as shown in Fig. 5, A and B. Fig. 6, C and D, show that sera from mice immunized with P1.17 or normal serum lacked antibodies binding to p97.

An Ab3 response was detected also in C3H/HeN mice after they had been immunized with Ab2 conjugated to KLH. Serum from the immunized C3H/HeN mice bound to soluble p97 antigen in a solid phase ELISA (Fig. 7). The binding seen with sera from mice getting Ab2 No. 3 was similar to that of MAb 96.5 when this was tested over a concentration of 1–0.004 ng/ml; this was significant (P < 0.05) when compared to control sera. Absorption with either 2a or par cells before the testing for binding to soluble p97 verified that the binding seen with sera from mice immunized with No. 3 or 7 was to p97.

Experiments were also done in which BALB/c and C3H/HeN mice were immunized with an Ab2 which had not been conjugated to KLH. Serum from these mice was found to contain antibodies which bound to Fab 96.5. To determine whether these antibodies were Ab2 which still remained in the circulation, or whether any Ab4 had been induced, an ELISA was performed with HRP conjugates which could identify not only IgG1 (the isotype of the Ab2) but also IgG2b and IgG3. The findings indicated that the mouse sera contained antibodies which could bind to the Ab1 and belonged to the IgG2b and IgG3 classes (data not shown).
One of the goals for making Ab2 is to obtain a therapeutically useful tumor vaccine. For this we believe that one should select Ab2 which can elicit cell-mediated immune responses, in view of the fact that antibodies Nos. 3, 5, and 7 functionally mimic p97 antigen. They do, however, not exclude alternative explanations such as steric hindrance. It became important, therefore, to investigate whether these three Ab2 could induce an immune response in mice. Since humoral antibody responses can generally be studied more easily and precisely than cell-mediated responses, we first searched for Ab3 which could bind to p97. This work was facilitated by the availability of a transfectected mouse melanoma line, 2a, which expresses a high level of p97, as well as its p97-negative “parent” (par).

To obtain an Ab1-like response in vivo, the conjugation of an Ab2 to KLH was necessary. When KLH was not used, the sera of the immunized mice contained both antibodies of the IgG1 isotype of the Ab2 and antibodies which were of other isotypes (IgG2b, IgG3) and may have been Ab4. If Ab4 were, indeed, generated, on Ab3 response may have occurred at some point. However, studies in another species are needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species controlled by Th genes but most likely was induced by the Ab2 acting as an antigen (25), although studies in another species needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species controlled by Th genes but most likely was induced by the Ab2 acting as an antigen (25), although studies in another species needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species controlled by Th genes but most likely was induced by the Ab2 acting as an antigen (25), although studies in another species needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species controlled by Th genes but most likely was induced by the Ab2 acting as an antigen (25), although studies in another species needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species controlled by Th genes but most likely was induced by the Ab2 acting as an antigen (25), although studies in another species needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species controlled by Th genes but most likely was induced by the Ab2 acting as an antigen (25), although studies in another species needed to definitively establish this. The Ab1-like Ab3 were acting as an antigen (25), although studies in another species.
of the importance of such responses in tumor rejection (26). We, therefore, investigated the ability of Ab2 Nos. 3 and 7 to induce cell-mediated immunity to p97 and concentrated on looking for DTH responses, which play a key role in tumor rejection (27). No DTH response to p97 was observed. In view of this, we were not surprised over our failure to detect any significant transplantation immunity to p97-positive tumor cells. This was in contrast to what was seen in mice immunized with v-p97NY, a recombinant anti-p97 vaccine, where strong DTH and effective protection to tumor was observed, as previously described (15, 24). Our data suggest that the epitope identified by MAb 96.5 is not a target for cell-mediated anti-p97 immunity leading to tumor rejection, although we are, of course, aware of difficulties in making conclusions from negative data. In contrast, a polyclonal antiidiotypic antiserum which had been raised against MAb 8.2, an antibody to a different epitope of p97, induced DTH, as well as antibodies to p97 (14).

Our findings indicate that monoclonal Ab2 obtained by immunization with monoclonal Ab1 can effectively induce Ab1-like Ab3 antibodies. Furthermore, Ab2 described in this paper are likely to be useful for a variety of purposes, such as development of assays for Ab1 and for screening various genetically modified anti-p97 antibodies, including chimeric (mouse-human) antibodies. A different strategy is probably needed, however, to obtain Ab2 that can effectively induce cell-mediated antitumor responses leading to tumor rejection. Perhaps a good approach is to use, as immunogen, clones of helper or cytolytic T-cells involved in the rejection of p97-positive melanoma cells by v-p97NY immunized hosts.

ACKNOWLEDGMENTS

We thank Stephanie Ashe for excellent technical assistance and Phyllis Harps for preparation of the manuscript.

REFERENCES

Monoclonal Antiidiotypic Antibodies Related to the p97 Human Melanoma Antigen

Maria Kahn, Ingegerd Hellström, Charles D. Estin, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/12/3157

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.