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Vaccination with a Human High Molecular Weight Melanoma-Associated Antigen Mimotope Induces a Humoral Response Inhibiting Melanoma Cell Growth In Vitro

Stefan Wagner,*† Christine Hafner,‡ Dorothee Allwardt,*† Joanna Jasinska,*† Soldano Ferrone,‖ Christoph C. Zielinski,¶ Otto Scheiner,¶ Ursula Wiedermann,‡ Hubert Pehamberger,¶ and Heimo Breiteneder2†¶

Peptide mimics of a conformational epitope that is recognized by a mAb with antitumor activity are promising candidates for formulations of anticancer vaccines. These mimotope vaccines are able to induce a polyclonal Ab response focused to the determinant of the mAb. Such attempts at cancer immunotherapy are of special interest for malignant melanoma that is highly resistant to chemotherapy and radiotherapy. In this study, we describe for the first time the design and immunogenicity of a vaccine containing a mimotope of the human high m.w. melanoma-associated Ag (HMW-MAA) and the biological potential of the induced Abs. Mimotopes were selected from a pVIII-9mer phage display peptide library with the anti-HMW-MAA mAb 225.28S. The mimotope vaccine was then generated by coupling the most suitable candidate mimotope to tetanus toxoid as an immunogenic carrier. Immunization of rabbits with this vaccine induced a specific humoral immune response directed toward the epitope recognized by the mAb 225.28S on the native HMW-MAA. The induced Abs inhibited the in vitro growth of the melanoma cell line 518A2 up to 62%. In addition, the Abs mediated 26% lysis of 518A2 cells in Ab-dependent cellular cytotoxicity. Our results indicate a possible application of this mimotope vaccine as a novel immunotherapeutic agent for the treatment of malignant melanoma. The Journal of Immunology, 2005, 174: 976–982.

Malignant melanoma affects an estimated 100,000 patients worldwide each year, and the incidence continues to increase (1–3). New therapeutic strategies are necessary because of the high resistance of malignant melanoma to chemotherapy and radiotherapy. Malignancy is often linked to melanoma-associated Ags (MAAs)3 that are highly expressed on melanoma cells as a result of qualitative and quantitative changes in the antigenic profile during malignant transformation of melanocytes (4). The identification and molecular characterization of these MAAs have provided well-defined targets for immunotherapy (5, 6).

One of these Ags, the human high m.w. MAA (HMW-MAA), also known as the human melanoma-associated chondroitin sulfate proteoglycan, is expressed by a large percentage of melanoma lesions with limited heterogeneity and restricted distribution in normal tissues (7). Its expression correlates with the increase of the proliferative capacity of melanoma cells (8). The HMW-MAA was identified with mAbs (3), including the mAb 225.28S (reviewed in Ref. 7). Besides its use in immunoscintigraphy (9) and in immunohistochemical assays (10), the mAb 225.28S has also been used for therapeutic purposes as immunocjugate with cytotoxic agents both in animal models (11, 12) and in patients with melanoma (13). Interestingly, a retrospective analysis of >300 patients with melanoma who underwent immunoscintigraphy with 99mTc-labeled F(ab′)2 of mAb 225.28S showed a prolongation of survival time (9). Active specific immunotherapy directed against the HMW-MAA was performed with the anti-Id mAb MF11-30 that mimics the epitope recognized by anti-HMW-MAA mAb 225.28S (14). In a clinical study of 19 melanoma patients, immunization with the anti-Id mAb MF11-30 was associated with complete remission in one patient and minor responses in three patients (15). Recently, we were able to show that the mAb 225.28S suppressed human melanoma tumor growth in SCID mice up to 50% in comparison with sham-treated mice (16).

A further development of antitumor strategies is the use of peptides that represent putative B cell epitopes and are designed from the primary amino acid sequence of tumor Ags (17, 18). The application of such peptides to induce an antitumor humoral immune response is preferable to other vaccines because peptides are easily synthesized, chemically stable, and free of undesired contaminants. However, it seems that B cell epitopes capable of inducing protective Ab responses are likely to be conformational in nature and, therefore, unlike linear epitopes cannot be predicted from an analysis of the primary amino acid sequence (19, 20). Phage display technology represents a valuable approach to develop vaccines for the induction of a defined Ab response directed toward a conformational epitope. By screening a mAb with a phage library displaying peptides, ligands of various lengths can be identified.
that mimic the epitope recognized by the mAb on the corresponding tumor Ag (21). Immunizations with these mimotopes elicit Abs against the Ag as it has been shown for the prostate-specific membrane Ag (22), an unknown Ag from fibrosarcoma cells (23) or tumor-associated carbohydrates (24). In the latter two cases, the induction of tumor Ag-specific Abs prolonged host survival in animal models.

In this study, we demonstrate that an HMW-MAA mimotope conjugated to tetanus toxoid (TT) represents an effective vaccine to elicit HMW-MAA-specific Abs in rabbits that inhibit melanoma cell growth in vitro.

Materials and Methods

Antibodies

The mAbs 225.28S, TP41.2, and TP61.5 and the anti-Id mAb MF11-30, which mimics the mAb 225.28S-defined determinant, were developed and characterized, as described elsewhere (14, 25, 26).

Biotinylation of mAbs

NHS-LC-Biotin (Pierce) was diluted in dimethylformamide at a concentration of 40 mg/ml. Five microliters of this solution was added to 1 mg/ml mAb in PBS and incubated for 45 min at room temperature. Excess NHS-LC-biotin was removed by dialysis against PBS.

Cell lines

The human melanoma cell line 518A2, which expresses high levels of HMW-MAA (a gift from P. Schrier, Leiden University Medical Center, Leiden, The Netherlands), was maintained in DMEM (Invitrogen Life Technologies). The human melanoma cell line M14 with no detectable expression of 40 mg/ml. Five microliters of this solution was added to 1 mg/ml cell lysate and incubated for 2 h at room temperature with 1 mg/ml cell lysate.

Preparation of cell lysates

A total of 6 × 10^7 melanoma cells was suspended in 2 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 1% Triton X-100, and 1× complete EDTA-free protease inhibitor mix (Roche), extensively vortexed, and incubated on ice for 15 min. After disruption, samples were centrifuged 10 min at 800 × g at 4°C. Supernatants were removed from cell debris and stored at −20°C until use. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce).

Phage display, affinity selection, and sequence analysis

Peptide ligands for the mAb 225.28S were selected from a pVIII-9mer phage display library composed of random 9 aa residues, displayed on filamentous phage as fusion to the NH2 terminus of the major coat protein pVIII. Three rounds of selection of ligands were performed in the laboratories of Schauer-N (Copenhagen, Denmark). In short, the mAb 225.28S was immobilized and incubated with the phage library. Phages displaying peptides that bound to the mAb 225.28S were eluted and amplified in Escherichia coli. After three rounds of selection, single clones that bound to the mAb 225.28S were identified by phage ELISA, and subsequently, DNA of inserts was sequenced.

Synthesis of peptides

The peptides TRTNPWPALGGGGGC (225D9.2), AEGEFTTRTPWPALGGGGGC (225D9.2), TRTQGRPFPQGGGFC (225D9.3), AEGEFTQGPFRGPQGGGFC (225D9.3), and an unrelated peptide derived from the oncoprotein Her-2/neu, PESFDGDPSANTALQPQGGGFC (17) (Pep1, 23aa), were synthesized using F-moc strategy by pICHEM. The purity of the peptides was >95%, as assessed by HPLC.

Inhibition ELISA using synthetic peptides

Ninety-six-well microtiter plates (Maxisorp; Nunc) were coated overnight at 4°C with 4 µg of mAb TP41.2 per ml coating buffer (50 mM NaHCO3, pH 9.6). One microgram of biotinylated mAb 225.28S was incubated with increasing concentrations of 0, 10, 100, and 500 µg/ml of synthetic peptides in TBS/0.5% v/v Tween 20 (TBST) containing 0.5% BSA overnight at 4°C. The next day, microtiter plates were blocked with TBST/3% milk powder and incubated for 2 h at room temperature with 1 mg/ml cell lysate diluted in TBST containing 1% BSA. After washing, mAb 225.28S pre-incubated with peptides was added, and incubation was continued for an additional 2 h at room temperature. Bound biotinylated mAb 225.28S was detected using alkaline phosphatase (AP)-conjugated streptavidin (Amer- sham Biosciences), followed by addition of p-nitrophenylphosphate (Sigma-Aldrich). Absorbance was measured at 405 nm. Percentage of inhibition was calculated as follows: 100 − (OD (inhibited)/OD (uninhibited) × 100).

Conjugation of peptides

Peptides were coupled to the carrier protein TT (Berna) or keyhole limpet hemocyanin (KLH; Sigma-Aldrich) using the heterobifunctional cross-linker reagent m-maleimidobenzoyl-N-hydroxysuccinimide (MBS; Pierce). The amino groups of the carrier proteins were first activated by a 30-min incubation at room temperature with a 25-fold molar excess of MBS. Excess MBS was removed by a desalting column (PD-10 column; Amersham Biosciences). In a second step, peptides were added in a molar ratio of 40:1 peptide-to-carrier protein. Cross-linking occurred to the cysteine residues on the peptides within 3 h at room temperature. After 3 h, an aliquot was collected to quantitate conjugation. Unbound peptides were removed by dialysis against PBS. The degree of conjugation was estimated using Ellman’s reagent (Pierce) with a standard curve with known quantities of cysteine.

Immunization

Immunizations with peptide conjugate 225D9.2-TT or the control conjugate Pepl-TT were performed in rabbits at Charles River Laboratories. Immunizations were done in three times in 14- to 21-days intervals, and each with 200 µg of peptide conjugates adsorbed to CFA and IFA. Seven days after the last immunization, the animals were sacrificed.

Purification of rabbit IgG Abs

Total IgG from sera of rabbits were purified using a HiTrap protein A HP column (Amersham Biosciences). Proteins of 20 ml of serum were precipitated with ammonium sulfate at 80% saturation. Precipitated proteins were resuspended in binding buffer (20 mM NaH2PO4, pH 7.0) and desalted using a PD-10 column. The HiTrap column was equilibrated with binding buffer, the protein sample applied, and the column washed with binding buffer. Bound Abs were eluted with 0.1 M citric acid, pH 3.0. Purification was monitored using nonreducing 8% SDS-PAGE, and protein concentration was determined with a bicinchoninic acid protein assay (Pierce).

ELISA

Peptide-specific Ab response. Ninety-six-well microtiter plates were coated with 5 µg/ml peptide 225D9.2 conjugated to KLH or 5 µg/ml KLH in 50 mM carbonate buffer, pH 9.6, overnight at 4°C. Non-specific binding sites were blocked with TBS/0.5% v/v Tween 20 (TBST) containing 3% milk powder. Purified Abs from the mimotope conjugate and the control peptide conjugate immunizations were diluted at concentration of 0.05, 0.1, 0.5, 1.5, and 10 µg/ml in TBST containing 0.5% BSA, added to Ag-coated plates, and incubated overnight at 4°C. After washing, bound Abs were detected using AP-conjugated swine anti-rabbit IgG (Dako-Cytomation) diluted 1/500 in TBST containing 0.5% BSA. Color development was performed by addition of p-nitrophenylphosphate. The absorbance was measured at 405 nm.

HMW-MAA-specific Ab response

Ninety-six-well microtiter plates were coated overnight at 4°C with 4 µg of mAb TP41.2 per ml coating buffer (50 mM NaHCO3, pH 9.6). Plates were blocked with TBST/3% milk powder and incubated for 2 h at room temperature with 1 mg/ml cell lysate diluted in TBST containing 1% BSA. Subsequently, plates were incubated at 4°C overnight with increasing concentrations (12.5, 25, 50, 100, and 200 µg/ml) of purified Abs from the mimotope conjugate and the control peptide conjugate immunizations diluted in TBST containing 1% BSA. Bound IgG was detected, as described above.

Ab response to the anti-Id MF11-30

Ninety-six-well microtiter plates were coated overnight at 4°C with the mAb MF11-30 or the isotype control mAb TP61.5 (4 µg/ml coating buffer, 50 mM NaHCO3, pH 9.6). After blocking with TBST/3% milk, plates were incubated overnight at 4°C with increasing concentrations (12.5, 25, 50, 100, and 200 µg/ml) of purified rabbit Abs diluted in TBST containing 1% BSA. Bound IgG was detected, as described above.
Inhibition of MF11-30 binding to the mAb 225D.28S

For inhibition experiments, microtiter plates were coated overnight at 4°C with the mAb 225D.28S (4 μg/ml coating buffer). Biotinylated mAb MF11-30 (12 ng/ml) was incubated overnight at 4°C with increasing concentrations (0, 125, 250, 500, 750, and 1000 μg/ml) of purified rabbit Abs from the mimotope conjugate and the control peptide conjugate immunizations diluted in TBST containing 1% BSA. The next day, plates were blocked with TBST/3% milk, and before incubation with the mAb MF11-30 was added. Incubation was continued for 2 h at room temperature. Bound biotinylated mAb MF11-30 was detected using AP-conjugated streptavidin, followed by addition of p-nitrophenylphosphate. The absorbance was measured at 405 nm. Percentage of inhibition was calculated as follows: 100 − (OD (inhibited)/OD (uninhibited)) × 100.

Inhibition of tumor cell growth in vitro

Tumor cells were plated in 96-well microtiter plates (Costar) at an optimal density for linear growth: 1.5 × 10^4 cells/well for the 518A2 cell line and 1 × 10^5 cells/well for the control cell line M14. Cells were allowed to adhere overnight at 37°C. Total IgG isolated from the mimotope conjugate and the control peptide conjugate immunizations was added at increasing concentrations (0, 10, 100, and 1000 μg/ml), and incubation was continued for an additional 72 h at 37°C. Cells were pulsed with 0.5 Ci of [3H]thymidine/well (PerkinElmer Life Sciences) for another 6 h at 37°C, and then harvested. Incorporated [3H]thymidine was measured in a 1205 Betaplate liquid scintillation counter (Wallac Oy). Percentage of inhibition of proliferation was calculated by comparing the cpm values of treated cells with those of untreated controls, which were set at 100%.

Ab-dependent cellular cytotoxicity (ADCC) assay

ADCC was determined using the CytoTox 96 assay (Promega) and performed according to manufacturer’s instructions with some modifications. Human PBMC were isolated from healthy donors by differential centrifugation on Ficoll-Paque density gradients, as described elsewhere (27), and used as effector cells. The target cell lines 518A2 and M14 were plated in 96-well plates at a density of 1 × 10^4 cells/well, and purified rabbit Abs were added at 50 μg/well. After 1 h of incubation at 37°C, effecter cells were added and the cocultures were incubated for another 4 h at 37°C. The E:T ratio was 150:1. Cytotoxicity was calculated as follows: percentage of lysis = ((experimental − target spontaneous)/(target maximum − target spontaneous)) × 100.

Results

Identification of peptide ligands for the mAb 225D.28S

For identification of peptides capable of binding to the mAb 225D.28S, the mAb was immobilized and incubated with a pVIII-9mer phage peptide library. After three rounds of selection, 200 single clones were tested by phage ELISA for their binding ability to the mAb 225D.28S. Forty-nine positive clones were subjected to DNA sequencing yielding two predominant DNA sequences that encoded the amino acid sequences TRTNPWPAL (225D9.2) and TRTQPGRFP (225D9.3). Eight phage clones displayed sequence 225D9.2, and seven phage clones sequence 225D9.3 (Table I). No similarity to the sequence of HMW-MAA could be observed by linear alignment using the BLAST search algorithm. Comparison of the two sequences with a described mimotope of HMW-MAA that was isolated by panning the mAb 225D.28S with a pIII-15mer phage library (28) showed a consensus sequence of TRTXP (Table I).

Binding characteristics

To investigate whether the phage-displayed peptides 225D9.2 and 225D9.3 were constrained by the structural context of the phage surface or the N-terminal extension of the pVIII coat protein of the phage was involved in mimotope formation, inhibition experiments were performed with synthetic peptides. Peptides 225D9.2 and 225D9.3 were synthesized with an additional glycine linker and a C-terminal cysteine residue for conjugation purpuses. Both peptides were also synthesized with additional 5 aa (AEGEF), termed 225D9.2" and 225D9.3", at the N terminus representing the N-terminal sequence of the pVIII coat protein. The ability of the peptides to inhibit the binding of the mAb 225D.28S to HMW-MAA was tested in ELISA. The Ag was purified from a 518A2 melanoma cell lysate by binding to anti-HMW-MAA mAb TP41.2. When biotinylated mAb 225D.28S was allowed to bind to HMW-MAA in the presence of increasing concentrations of peptides, a dose-dependent inhibition of binding up to 63 and 34% was observed for peptides 225D9.2" and 225D9.3", respectively (Fig. 1). Interestingly, binding of the mAb could not be diminished by peptides 225D9.2 and 225D9.3 (data not shown). Therefore, the 5-aa AEGERF representing the N-terminal portion of the pVIII protein of phage are part of the mimotopes. The unrelated peptide Pep1 also did not influence the binding characteristics of mAb 225D.28S.

Immunogenicity of peptide 225D9.2" in rabbits

Peptide 225D9.2" was coupled to TT or KLH as carrier protein, and the reaction was monitored using biotinylated 225D.28S in dot blot and ELISA experiments (data not shown). A conjugation ratio of 12–15 peptides per molecule TT could be determined by the Ellman’s reagent. Rabbits were immunized with the conjugate TT-225D9.2" or the control conjugate TT-Pep1. After three immunizations, Abs were purified from sera using a HiTrap protein A HP column. Twenty milliliters of serum yielded ~15 mg of IgG Abs. The purity was greater than 95%, as confirmed by SDS-PAGE (data not shown). Peptide-specific Abs were determined by ELISA after incubation of coated peptide-KLH conjugate or KLH with purified total IgG Abs. The 225D9.2"-specific Abs were already weakly detectable at a concentration of 0.1 μg/ml, and high peptide-specific Ab levels were measured at concentrations of 0.5 μg/ml and more (Fig. 2). In contrast, Abs purified from the TT-Pep1-immunized rabbit did not bind to the peptide. No Abs directed against KLH could be determined.

The HMW-MAA-specific immune response was evaluated in ELISA testing the induced Abs with HMW-MAA purified from a melanoma cell lysate by binding to the coated mAb TP41.2. HMW-MAA-specific Abs were detectable among the purified Abs following the immunization with TT-225D9.2" at a concentration of 12.5 μg/ml and more, but not among Abs purified following the

Table I. Insert sequences of phages displaying ligands for mAb 225D.28S

<table>
<thead>
<tr>
<th>Phage Clone</th>
<th>Sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>225D9.2</td>
<td>TRTNWPAL</td>
<td>8 phage clones</td>
</tr>
<tr>
<td>225D9.3</td>
<td>TRTPQRGF</td>
<td>7 phage clones</td>
</tr>
<tr>
<td>15-aa clone (28)</td>
<td>TQTVTRTPWGLEPPK</td>
<td></td>
</tr>
</tbody>
</table>
immunization with the control conjugate TT-Pep1 or when using a cell lysate of the HMW-MAA-negative melanoma cell line M14 (Fig. 3).

**Anti-Id MF11-30-specific Abs**

Abs specific for the epitope recognized by the mAb 225.28S were determined by ELISA using the mAb MF11-30, which is the anti-Id to mAb 225.28S. Purified Abs derived from immunization with TT-225D9.2 + specifically bound to mAb MF11-30 already at a concentration of 12.5 μg/ml (Fig. 4). The Ab response following the immunization with the control conjugate TT-Pep1 was in the range of the response of the purified Abs against the isotype control, the anti-HMW-MAA mAb TP61.5. To ensure that the MF11-30-specific Abs are directed toward the same epitope as the mAb 225.28S, inhibition of the binding of the anti-Id MF11-30 to the mAb 225.28S by the induced Abs was investigated. The purified Abs from the immunization with TT-225D9.2 + were able to inhibit the binding of the anti-Id up to 47% in a dose-dependent manner (Fig. 5). No inhibition was detectable using the purified Abs from the immunization with TT-Pep1 (Fig. 5).

**Biological activity of induced Abs**

**Tumor cell growth inhibition in vitro.** We used a [3H]thymidine proliferation assay to assess the tumor growth inhibition of the HMW-MAA-specific Abs. The melanoma cell lines 518A2 (HMW-MAA*positive (pos)) and M14 (HMW-MAA*negative (neg)) were incubated with increasing concentrations of purified Abs (Fig. 6). The inhibition of tumor cell proliferation, measured by incorporation of [3H]thymidine into 518A2 cells, was increased in a dose-dependent manner by purified Abs. At a concentration of 100 μg/ml anti-225D9.2 + Abs, cell proliferation was inhibited by 19%. This inhibition was increased up to 62% when 1000 μg/ml Abs were used. Incubation with anti-Pep1 Abs at identical concentrations did not influence proliferation of 518A2 cells. Anti-Pep1 as well as anti-225D9.2 + Abs inhibited proliferation of M14 cells ~20% when used at a concentration of 1000 μg/ml. The data presented are mean values of three independent experiments.

**ADCC**

Abs purified from rabbits immunized with TT-225D9.2 + or TT-Pep1 were examined for their ability to mediate tumor cell lysis in an ADCC-dependent manner (Fig. 7). Incubation of the melanoma cell line 518A2 (HMW-MAA*pos) with the purified Abs from the rabbit immunized with the 225D9.2 +-TT conjugate induced 26% cell lysis. Abs purified from the serum of a rabbit immunized with the control conjugate TT-Pep1 mediated only 7% cell lysis, and the Abs obtained from immunizations with TT-225D9.2 + or TT-Pep1 conjugates mediated cell lysis of the melanoma cell line M14 (HMW-MAA*neg) at 10 and 6%, respectively. The data presented are mean values of three independent experiments.

**Discussion**

One of the major goals of cancer immunotherapy is the generation of vaccines capable of eliciting protective immunity against tumors. Peptides mimicking epitopes of clinically relevant mAbs conjugated to an immunogenic carrier protein could be used as vaccines to actively induce a polyclonal antitumor Ab response. Mimotope technology is able to define such peptides and has the major advantage to allow the formulation of vaccines based on conformational epitopes without the knowledge of their structure. In the present study, we examined a strategy to design and test such a mimotope vaccine for the induction of a humoral immune response directed against the HMW-MAA, an Ag expressed in a large number of melanoma lesions with limited intra- and interlesional heterogeneity (29). Mimotopes were selected from a random phage peptide library by affinity screening with the mAb 225.28S that is directed to the HMW-MAA (25). Immunization of rabbits with the most suitable mimotope coupled to TT induced an Ab response directed toward the synthetic mimotope and cross-reactive with the epitope of the mAb on the native melanoma Ag. In biological in vitro assays, the induced Abs inhibited melanoma tumor cell growth in a direct and indirect mechanism as tested by inhibition of cell proliferation and ADCC.

The clinical efficacy of mAbs directed against tumor Ags suggests that the induction of a strong and targeted humoral immune response may be biologically important. One of the best characterized and studied Abs is mAb 4D5, which has shown specific
inhibition of ErbB2-overexpressing tumor cells, and its humanized form, Herceptin, is currently in use for therapy of breast cancer (30–34). For melanoma, Bender et al. (9) have observed in a retrospective analysis of >300 patients with melanoma that those who underwent immunoscintigraphy with 99mTc-labeled F(ab’)2 of the mAb 225.28S showed prolonged survival times. The anti-Id mAb MF11-30 that mimics the epitope defined by anti-HMW-MAA mAb 225.28S was used as an immunogen for active immunotherapy, and a significantly prolonged survival was observed in patients who developed a high titer of anti-Id Abs (35). In addition, the mAb 225.28S was shown to inhibit human melanoma tumor growth in SCID mice (16). However, currently, there is no mAb in routine clinical use for passive immunotherapy of malignant melanoma.

Based on the data of its potential clinical efficacy and its tumor-inhibitory activity in a human xenotransplant model (9, 15), we decided to use the mAb 225.28S for the generation of a mimotope vaccine for the induction of a humoral immune response directed against the HMW-MAA. Affinity screening of this mAb with a peptide phage library yielded the two predominant sequences 225D9.2 and 225D9.3 (Table I). It is of interest to note that they shared a 5-aa-residue motif TRTXTP with a mimotope described for the mAb 225.28S isolated from a 15mer phage peptide library (Table I). It is known that the interaction between the Ab and phage-displayed peptide depends on the peptide’s microenvironment that is provided by the phage particle during the affinity selection (36). Peptides were synthesized with and without additional 5 aa residues at their NH2 terminus that resulted from the fusion of the peptides with the phage coat protein. By adding the 5 residues of the NH2 terminus of the phage coat protein, we attempted to obtain peptides that matched the original situation of the mimotope on the phage more closely. In the inhibition ELISA, only the peptides with the additional 5 aa residues were able to inhibit the binding of the mAb 225.28S to the HMW-MAA (Fig. 1). These data show that in our case the short additional N-terminal portion of the phage coat protein was indeed involved in mimotope formation. In contrast, it also seems possible that the short portion of the phage coat protein assisted the presentation of the peptide or modified its conformation to duplicate that in the native tumor Ag.

Peptide 225D9.2+ showed the highest capacity to inhibit the binding of the mAb 225.28S to the HMW-MAA, and was therefore coupled to the immunogenic carrier protein TT. The conjugate TT-225D9.2+ was specifically recognized by the mAb 225.28S, whereas no reactivity of the mAb was observed to the control conjugate TT-Pep1 (data not shown). Immunization of rabbits with the mimotope-TT conjugate resulted in the induction of mimotope-specific Abs. The specificity was measured in an ELISA using an immobilized conjugate consisting of the mimotope and KLH as an unrelated carrier molecule (Fig. 2). In this assay, the mimotope was detected by specific Abs present in nanogram quantities of total purified Abs. A mimotope-based vaccine strategy will only be effective if the generated mimotope-specific response allows recognition of the native protein. We used an established HMW-MAA-catching ELISA to measure the Ag-specific humoral response (37). In this assay, HMW-MAA-specific Abs were detected in microgram quantities of total purified Abs. The binding was specific, because Abs from the immunization with the unrelated peptide conjugated to TT did not react, and no Ab binding was observed to a lysate of M14 melanoma cells (HMW-MAAneg) (Fig. 3).

To be useful as a vaccine component, a mimotope is required to mimic the antigenicity of the epitope by binding to the Ab that recognizes the native epitope. It also needs to mimic the immunogenicity of the epitope by inducing Abs cross-reactive with the native epitope. The measurement of HMW-MAA-specific Abs indicates that the degree of mimicry afforded by our peptide was sufficient to induce anti-peptide Abs that cross-reacted with the original melanoma Ag. Vaccination with such immunogenic mimotopes has been shown in several studies to result in disease protection, especially in the field of viral infections (38–42), although other studies were less promising (43, 44). To our knowledge, there is only one report about a mimotope vaccine in the field of oncology that shows the induction of a humoral immune response to a protein epitope of an unknown Ag from fibrosarcoma cells and the subsequent reduction of the growth of metastasis (23).

One of the presumed benefits of mimotopes is their ability to direct the immune response toward a specific epitope. Whether our induced anti-HMW-MAA Abs recognize the same epitope as the mAb 225.28S was investigated using the anti-Id mAb MF11-30. The anti-Id was specifically recognized by the induced Abs, and binding of the mAb MF11-30 to the mAb 225.28S was specifically inhibited by the induced Abs (Figs. 4 and 5). From these data, we conclude that our induced polyclonal immune response contains Abs directed toward the epitope of the mAb 225.28S.

Epitope specificity is of high importance for the success of a mimotope vaccine. The capacity to narrowly focus the immune
response to a desired epitope is of particular relevance, as interaction of an Ab with a tumor Ag can also have the potential of stimulating tumor growth (45). In biological in vitro assays, we could demonstrate that our induced Abs were able to inhibit the growth of melanoma cells up to 62% and mediated 26% lysis of HMW-MAA-overexpressing 518A2 melanoma cells. This is to our knowledge the first example in which tumor Ag-specific Abs induced by a mimotope vaccine were shown to inhibit tumor cell growth in vitro.

In summary, we have selected a mimotope of the HMW-MAA that is equivalent to the epitope that is recognized by the mAb 225.28S. The mimotope, coupled to TT, is able to induce an anti-HMW-MAA immune response in rabbits after immunization. The induced Ab response is able to inhibit melanoma tumor cell growth in vitro. These exciting results suggest that our mimotope vaccine could be used as a new strategy for the treatment of malignant melanoma.

References


