Human High Molecular Weight–Melanoma Associated Antigen Mimicry by Mouse Anti-Idiotypic Monoclonal Antibody MK2-23: Modulation of the Immunogenicity in Patients with Malignant Melanoma

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ABSTRACT

The mouse anti-idiotypic (anti-id) mAb MK2-23 bears the mirror image of the antigenic determinant defined by antihuman high molecular weight–melanoma associated antigen (HMW-MAA) mAb 763.74. The purpose of this study was to evaluate the effect of conjugation to a carrier and administration with an adjuvant on the immunogenicity of anti-id mAb MK2-23 in patients with malignant melanoma and to analyze the relationship between development of humoral immunity and survival time of patients. Fifty-eight patients were sequentially entered into four immunization protocols which included administration of mAb MK2-23, mAb MK2-23 conjugated to keyhole limpet hemocyanin (KLH) and mixed with Bacillus Calmette-Guérin (BCG), mAb MK2-23 and CTX, and mAb MK2-23 conjugated to KLH and mixed with BCG and CTX. Six patients could not be evaluated since they withdrew from the clinical trial after the first immunization. Sera were tested for the development of anti-anti-id antibodies, including those reacting with HMW-MAA. Testing of sera for development of antimouse Ig antibodies was used to monitor the immune competence of patients. Conjugation to KLH and administration with BCG markedly enhanced the ability of mAb MK2-23 to induce anti-anti-id antibodies, including those reacting with HMW-MAA. In contrast, pretreatment with CTX had no detectable effect on the ability of mAb MK2-23 to elicit a humoral anti-anti-id response. Kaplan-Meier survival analysis showed that the performance status of patients, anti-anti-id antibody level, and development of anti-HMW-MAA antibodies had an effect on survival time. This effect was found when the survival time was calculated both from the day of the first immunization and from 4 weeks after the first immunization to the end of the study. A multivariate analysis by Cox regression showed that the development of anti-HMW-MAA antibodies was the most important variable for predicting survival, and that performance status was the only variable that significantly added to the prediction of survival. These data have to be interpreted with caution because of the retrospective nature of the analysis. Nevertheless, the present study suggests that mAb MK2-23 represents a useful immunogen to implement active, specific immunotherapy in patients with malignant melanoma.

INTRODUCTION

A number of studies in animal model systems has shown that anti-id antibodies which bear the internal image of tumor-specific antigens can induce immunity to the nominal tumor-specific antigen, and that the immune response can prevent or suppress the growth of tumors (for review, see Refs. 1 and 2). These findings have been recently paralleled by similar results in patients with malignant melanoma (3). Administration with a carrier and an adjuvant of the mouse anti-id mAb MK2-23, which bears the internal image of the antigenic determinant defined by antihuman HMW-MAA mAb 763.74, induced anti-HMW-MAA antibodies in about 60% of 23 patients with malignant melanoma. The development of humoral anti-HMW-MAA immunity was associated with a significant survival prolongation (3). These findings in conjunction with the lack of major side effects associated with repeated administrations of mouse anti-id mAb MK2-23 in spite of the development of high levels of antimouse Ig antibodies have prompted us to continue our investigations with anti-id mAb MK2-23 in a larger number of patients with malignant melanoma.

Previous studies have shown that individually a carrier and an adjuvant have no marked effect on the immunogenicity of the anti-id mAb MK2-23 in BALB/c mice and rabbits, whereas their combined use markedly enhances it (4, 5). Rabbits express HMW-MAA in their normal tissues with a distribution similar to that in humans (6). Therefore, the immune response of rabbits to anti-id mAb MK2-23 is likely to predict that of patients with malignant melanoma. In the present study, we have investigated the effect of conjugation with KLH and administration with BCG on the ability of anti-id mAb MK2-23 to elicit humoral immunity in patients with malignant melanoma.

1 The abbreviations used are: anti-id, anti-idiotypic; BCG, Bacillus Calmette-Guérin; CTX, cytotoxin; HMW-MAA, high molecular weight–melanoma associated antigen; ICAM-1, intercellular adhesion molecule 1; KLH, keyhole limpet hemocyanin; PS, performance status.

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2 To whom requests for reprints should be addressed.
anti-HMW-MAA immunity in patients with advanced malignant melanoma. Furthermore, we have investigated the effect of pretreatment with a low dose of CTX on the immunogenicity of anti-id mAb MK2-23 in patients with advanced malignant melanoma, since pretreatment with low doses of CTX has been reported to enhance the cellular and humoral immune response to tumor-associated antigens (7–10) by selectively inactivating some subsets of suppressor cells (11–13). We have correlated the development of humoral anti-HMW-MAA immunity with patient survival to determine the effect of anti-anti-id response on the clinical course of the disease.

MATERIALS AND METHODS

Patients. The study population consisted of 58 patients with American Joint Committee on Cancer stage III and IV melanoma. This number included the 23 patients who have been described in a previous article (3). Patients entered into these studies were required to have a biopsyclar proven malignant melanoma. Patients had to be off all therapies for at least 4 weeks prior to entry into these trials. They were required to have a Karnofsky PS of at least 60%, a normal liver function with bilirubin ≤ 2 mg/dl, serum creatinine ≤ 2 mg/dl, a WBC count ≥ 3000 cells/mm³, and a platelet count ≥ 100,000 cells/mm³. All patients were required to have measurable disease by physical examination, X-ray, computerized axial tomographic scan and/or magnetic resonance imaging, and a life expectancy of at least 3 months. Tumor burden was measured by assigning a score of 1 to skin lesions, 2 to lesions in lymph nodes, 3 to lesions in bone, lung, and genitourinary tract, and 4 to lesions in the brain, gastrointestinal tract, and liver. Patients with multiple metastases were assigned a tumor burden score corresponding to the worst prognosis. Informed consent was obtained from each participating patient.

Sentinel lesions were measured monthly. Complete blood counts were obtained weekly, and liver function tests were performed monthly. Serum was obtained weekly and stored at −20°C for analysis. Response was defined according to the guidelines of the National Cancer Institute (Bethesda, MD), as described elsewhere (14).

Cell Lines. Cultured human melanoma cells Colo 38 and M14/13, cultured human B lymphoid cells L14, which are autologous to M14/13 cells, and cultured human B lymphoid cells LG-2 were grown in medium RPMI 1640 supplemented with 10% serum plus (JRH Biosciences, Lenexa, KS) and 2 mm L-glutamine.

mAbs and Conventional Antisera. The mouse mAbs 149.53, an IgG1, 225.28, an IgG2a, and 763.74, an IgG1, to distinct and spatially distant determinants of HMW-MAA, the anti-ICAM-1 mAb CL207.14, an IgG1, and the anti-id mAbs MF11–30 and MK2-23, both IgG1, elicited with mAbs 225.28 and 763.74, respectively, were developed and characterized as described (15–18). Mouse mAbs were purified from ascitic fluid by sequential precipitation with caprylic acid and ammonium sulfate (19). The purity of mAb preparations was monitored by SDS-PAGE (20).

Affinity-purified goat anti-human IgG + M antibodies, affinity-purified goat anti-human IgG antibodies, affinity-purified goat anti-human IgM antibodies, and affinity-purified goat anti-mouse IgG Fc antibodies were purchased from Jackson ImmunoResearch Laboratories (Avondale, PA).

Antibodies were labeled with 125I utilizing the iodogen method (21).

Immunization Schedule with Anti-id mAb MK2-23. Pretreatment with CTX was performed by giving i.v. infusion of CTX (300 mg/m²) in 250 ml 5% dextrose/water, over 20 min, on days 0 and 7. Patients were immunized on days 4 and 11 with s.c. injections of anti-id mAb MK2-23 (2 mg/injection) without KLH and BCG, or of anti-id mAb MK2-23 conjugated to KLH and mixed with 0.1 ml (1 × 10⁷ organisms) of Tice BCG (Organon, West Orange, NJ). On day 32 patients were immunized with either mAb MK2-23 without KLH and BCG or with mAb MK2-23 conjugated to KLH. Additional injections were given if the titer of anti-anti-id antibodies which inhibit the binding of 125I-labeled mAb MK2-23 to mAb 763.74 by 90% was not at least 1:8 on day 35, and whenever the titer fell to less than 1:8 in the following weeks.

Serological Assays. The binding assay to measure human anti-mouse Ig antibodies and the inhibition assay to measure human anti-anti-id antibodies were performed in 96-well U-bottomed polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, VA) as described (14). The binding assay to measure antibodies reacting with human cells and with purified HMW-MAA was performed in 96-well U-bottomed Falcon flexible microtiter plates (Becton Dickinson Labware, Oxnard, CA) as described (14). Absorption was performed by incubating a patient’s serum with cells (1 ml serum/4 × 10⁸ cells) for 4 h at 4°C on a rotator. Serum was then harvested by centrifugation.

Immunoenchemical Methods. Anti-id mAbs were crosslinked to KLH (Sigma Chemical Co., St. Louis, MO) at the ratio of 1:1 (w/w) utilizing glutaraldehyde (22). Mouse IgG mAbs were conjugated to Affigel 10 beads (Bio-Rad Laboratories, Richmond, CA) at the concentration of 20 mg/ml gel following the manufacturer’s instructions. Anti-mouse IgG antibodies were removed from sera of patients by passage through a column of insolubilized mouse IgG. Absorption of human anti-mouse IgG antibodies from serum was monitored by testing its reactivity with unrelated mouse IgG mAbs in a binding assay with 125I-labeled anti-human IgG + M xenon antibodies.

Solubilization of melanoma cells with NP40 and purification of HMW-MAA by binding to anti-HMW-MAA mAb-coated microtiter plates were performed as described elsewhere (14).

Statistical Analysis. The Kaplan-Meier product limit method (23) and the log rank test (24) were used in the univariate analysis to assess the ability of individual prognostic variables to predict survival time. Survival time was calculated either from the day of the first immunization or from 4 weeks after the first immunization to the end of the study. The latter approach was used to eliminate the possibility of introducing a lead-time bias into the statistical analysis (25), since development of anti-HMW-MAA antibodies is a treatment outcome variable and not a pretreatment variable. The Cox proportional hazards regression model (26) was used in the multivariate survival analysis to identify a subset of variables, including any interactions between variables, that jointly predict survival. Likelihood ratio tests were used to assess the prognostic signif-

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icance of the multivariate factors. Diagnostic hazards plots were performed whenever feasible to ascertain the appropriateness of the proportional hazards assumption.

RESULTS

A total of 58 patients were sequentially entered into four immunization protocols. There was no prior selection of patients into any of the groups. Patients who met entry criteria were entered into the trial that was open at the time of evaluation. Patients were not prospectively randomized, since at the time the study was performed there was no evidence to suggest that any of the immunization protocols is superior to the other ones. Six patients could not be evaluated since they withdrew from the clinical trial after the first immunization. Of the remaining 52 patients, 33 were males and 19 females with a median age of 56 (range, 22–84) years and a median PS of 70% (range, 60–100%). Twelve patients had not received prior therapy for metastatic disease; the remaining patients had been treated with chemotherapy, immunotherapy, radiotherapy, and/or surgery.

The 52 patients were classified into four groups according to the trial they entered sequentially. In group 1, 12 patients were immunized with mAb MK2-23 without KLH and BCG. In group 2, 23 patients were immunized with mAb MK2-23 conjugated to KLH and mixed with BCG. These patients, who had been previously described (3), are included in the present study for comparison purposes. In group 3, 7 patients were pretreated with CTX and immunized with mAb MK2-23 without KLH and BCG. In group 4, 10 patients were pretreated with CTX and immunized with mAb MK2-23 conjugated to KLH and mixed with BCG. Among the patients in group 1, 1 patient had a tumor burden score of 2, 5 patients a score of 3, and 6 patients a score of 4. Among the patients in group 2, 4 patients had a tumor burden score of 2, 5 patients a score of 3, and 14 patients a score of 4. Among the patients in group 3, 1 patient had a tumor burden score of 2, 3 patients a score of 3, and 3 patients a score of 4. Among the patients in group 4, 2 patients had a tumor burden score of 2, 3 patients a score of 3, and 5 patients a score of 4. It is noteworthy that in terms of age, sex, PS, tumor burden, and prior therapy, the 17 patients who received CTX pretreatment did not differ from the 35 who did not. As far as these parameters are concerned, no statistically significant difference was found between the 33 patients who received mAb MK2-23 conjugated to KLH and mixed with BCG and the 19 patients who received mAb MK2-23 without KLH and BCG. The number of immunizations per patient ranged between 3.0 and 6.0 with an average of 4.3 in group 1, between 3.0 and 12.0 with an average of 5.7 in group 2, between 3.0 and 9.0 with an average of 6.0 in group 3, and between 3.0 and 9.0 with an average of 5.3 in group 4. The mean minimum amount of anti-id mAb MK2-23 required to induce a level of anti-anti-id antibodies sufficient to inhibit the binding of 125I-labeled anti-anti-id mAb MK2-23 to anti-HMW-MAA mAb 763.74 by 90% was 10.0, 7.3, 10.6, and 8.6 mg/patient in groups 1, 2, 3, and 4, respectively. The mean minimum time required to induce anti-anti-id antibodies was 9.0, 7.6, 7.3, and 6.3 weeks in groups 1, 2, 3, and 4, respectively. Although all of the immunized patients developed anti-mouse Ig antibodies, only between 50 and 78% in the four groups of immunized patients developed anti-anti-id antibodies (Table 1).

CTX had no detectable effect on the ability of anti-id mAb MK2-23 to induce anti-anti-id antibodies: they were developed by 9 (52%) of the 17 patients who were pretreated with CTX prior to immunization with mAb MK2-23 and by 24 (68%) of the 35 patients who were immunized with anti-id mAb MK2-23 without prior treatment with CTX. However, conjugation with KLH and administration with BCG significantly increased the ability of mouse anti-id mAb MK2-23 to induce anti-anti-id antibodies. The latter were developed by 24 (72%) of the 33 patients immunized with mAb MK2-23 conjugated to KLH and mixed with BCG, but only by 9 (47%) of the 19 patients immunized with mAb MK2-23 without KLH and BCG.

Preimmune sera from all of the patients displayed a low reactivity with HWM-MAA-bearing cultured human melanoma cells M14/13 and with the autologous cultured B lymphoid cells L14, which do not express HWM-MAA. The reactivity of preimmune sera with melanoma cells M14/13 was undetectable following absorption with cultured B lymphoid cells L14. The mean minimum amount of anti-id mAb MK2-23 required for a 2-fold increase in the level of antibodies reacting with melanoma cells was 12.0, 7.4, and 8.6 mg/patient in groups 1, 2, and 4, respectively. The mean minimum time required for a 2-fold increase in the level of antibodies reacting with melanoma cells was 12.0, 8.4, and 13.0 weeks in groups 1, 2, and 4, respectively. Examples of the kinetics of the development of antibodies reacting with melanoma cells in patients in groups 1, 2, and 4 are shown in Fig. 1. The characteristics of the reactivity with melanoma cells of sera from patients following immunization with mAb MK2-23 are summarized in Table 1. Sera with increased reactivity with cultured melanoma cells following immunization with mAb MK2-23 also displayed an increased reactivity with cultured B lymphoid cells (Fig. 1). However, the reactivity of the sera with cultured B lymphoid cells was markedly lower than that with cultivated melanoma cells. Furthermore, absorption of sera with cultured B lymphoid cells L14 completely removed the reactivity with the absorbing cells, but did not markedly affect that with the autologous cultured melanoma

### Table 1

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* Figures show the number of patients who developed anti-anti-id antibodies/total number of immunized patients.

** Mean titer of anti-anti-id antibodies that inhibit the binding of 125I-labeled anti-anti-id mAb MK2-23 to anti-HMW-MAA mAb 763.74 by 90%.

*** Range of the titer of anti-anti-id antibodies that inhibit the binding of 125I-labeled anti-anti-id mAb MK2-23 to anti-HMW-MAA mAb 763.74 by 90%.

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Immunotherapy of Melanoma with Anti-id mAb

Fig. 1  Kinetics of the development of antibodies reacting with cultured human melanoma cells M14/13 in patients M. M., M. S., T. A., and B. S. immunized with anti-id mAb MK2-23. In the weeks indicated by arrows, patients M. M. (A) and M. S. (B) were immunized with anti-id mAb MK2-23 (2 mg/injection) without KLH and BCG, and patients T. A. (C) and B. S. (D) were immunized with anti-id mAb MK2-23 (2 mg/injection) conjugated to KLH. The first two immunizations to the latter two patients were given with the immunogen mixed with BCG. In addition, patients M. S. (B) and B. S. (D) received CTX (300 mg/M2) on days 0 and 7. Sera were drawn in the indicated weeks. Sera (50 μl/well) were incubated with melanoma cells M14/13 (2 x 10^5 cells/50 μl PBS) (EI) for 2 h at 4°C. Following three washings with PBS, cells were incubated with 125I-labeled antihuman IgG + M xenonantibodies (1 X 10^5 cpm/well) for 2 h at 4°C. Cells were then washed five times with PBS. Bound radioactivity was measured in a gamma counter. Results are expressed as bound cpm/2 X 10^5 cells. Cultured human B lymphoid cells L14 (□), which are autologous to melanoma cells M14/13, were used as controls.

cells M14/13 (Fig. 2). The antibodies reacting with melanoma cells in sera absorbed with lymphoid cells are both IgG and IgM.

Additional experiments were then performed to prove that the sera from the immunized patients contain anti-HMW-MAA antibodies. To this end, sera were first incubated with an excess of mouse Ig to block anti-mouse Ig antibodies, and then were tested for reactivity with HMW-MAA purified from a melanoma cell extract by binding to a microtiter plate coated with anti-HMW-MAA mAb 149.53. The latter recognizes a determinant distinct and spatially distant from that recognized by mAb 763.74 (16). As shown in Fig. 3, serum from patient A. M. reacted with purified HMW-MAA. The reactivity is specific, since serum did not bind to ICAM-1 purified from a melanoma cell extract by binding to anti-ICAM-1 mAb CL207.14-coated plates. It is noteworthy that sera from patients did not react with HMW-MAA bound to mAb 763.74. The latter results suggest that sera from patients recognize the same (or spatially close) determinant as that recognized by mAb 763.74 (Fig. 3). This interpretation is consistent with the specific inhibition by sera of the binding of 125I-labeled mAb 763.74 to melanoma cells (Fig. 4). The anti-HMW-MAA antibodies induced by mAb MK2-23 express the corresponding idiotope in their antigen combining site, since mAb MK2-23 inhibited the binding to melanoma cells of sera from patients in a dose-dependent fashion. The inhibition is specific, since the binding of sera to melanoma cells was not affected by the unrelated anti-id mAb MF11-30 (Fig. 5).

Furthermore, mAb MK2-23 did not affect the binding to melanoma cells of sera from patients immunized with the anti-id mAb MF11-30.

Administration of mAb MK2-23 was not associated with side effects except for the occurrence of erythema and ulceration (sterile abscess) at the sites of injection, fever, chills, arthralgias, and myalgias in patients immunized with mAb MK2-23 conjugated to KLH and mixed with BCG.

The median survival of the 52 patients entered into these studies was 19 (range, 4–96) weeks. Kaplan-Meier survival analysis investigated the univariate effect of several variables, including tumor burden, PS, prior chemotherapy, CTX pretreatment, carrier and adjuvant utilization, anti-anti-id antibody level, and anti-HMW-MAA antibodies development. PS had an effect on survival, since the 24 patients with a PS greater than 70% had a significantly (P = 0.01) longer survival than the 28 patients with a PS of 70% or less. In contrast, neither tumor burden nor prior chemotherapy had an effect on survival.
Fig. 2  Specificity of antibodies reacting with cultured human melanoma cells M14/13 in patients M. M., M. S., T. A., and B. S. immunized with anti-id mAb MK2-23. In the weeks indicated by arrows, patients M. M. (A) and M. S. (B) were immunized with anti-id mAb MK2-23 (2 mg/injection) without KLH and BCG, and patients T. A. (C) and B. S. (D) were immunized with anti-id mAb MK2-23 conjugated to KLH. The first two immunizations to the latter two patients were given with the immunogen mixed with BCG. In addition, patients M. S. (B) and B. S. (D) received CTX (300 mg/M$^2$) on days 0 and 7. Sera were drawn in the indicated weeks. Following absorption with cultured human B lymphoid cells (100 μl/4 × 10$^6$ cells), sera (50 μl/well) were incubated with melanoma cells M14/13 (2 × 10$^6$ cells/50 μl PBS; □) for 2 h at 4°C. Following three washings with PBS, cells were incubated with 1251-labeled antihuman IgG + M xenoantibodies (1 × 10$^4$ cpm/well) for 2 h at 4°C. Cells were then washed five times with PBS. Bound radioactivity was measured in a gamma counter. Results are expressed as bound cpm/2 × 10$^3$ cells. Cultured human B lymphoid cells L14 (●), which are autologous to melanoma cells M14/13, were used as controls.

patients with a tumor burden score of 2 or less had a slightly longer survival than 42 patients with a tumor burden score higher than 2; the difference, however, was only significant at $P = 0.08$. The survival of 22 patients who had received prior chemotherapy was not significantly different from that of 30 who had not received it. Administration of CTX had little effect on survival, since the survival of 17 patients who were treated with CTX was not significantly different from that of 35 patients who were not pretreated with CTX. In contrast, conjugation of mAb MK2-23 to KLH and administration with BCG had a marked effect on survival: 33 patients who were immunized with mAb MK2-23 conjugated to KLH and mixed with BCG survived significantly ($P = 0.008$) longer than 19 patients who were immunized with mAb MK2-23 without KLH and BCG. This correlation is likely to reflect the enhancement by the carrier and adjuvant of the immunogenicity of mAb MK2-23, since the anti-anti-id antibody level and development of anti-HMW-MAA antibodies had a marked effect on survival. Thirty-three patients who developed anti-anti-id antibodies with a titer of at least 1:8 survived significantly ($P = 0.001$) longer than 19 patients who did not. Similarly, 18 patients who developed anti-HMW-MAA antibodies survived significantly ($P = 0.001$) longer than 34 patients who did not.

A multivariate analysis by Cox regression showed that the development of anti-HMW-MAA antibodies was the most important factor for predicting survival, and that PS was the only variable that significantly ($P = 0.0005$) added to the prediction of survival in these patients. A slight, but not statistically significant interaction between humoral anti-HMW-MAA immunity and PS was detected. A separate multivariate analysis of the 34 patients who did not develop anti-HMW-MAA antibodies did not identify any predictor for survival, including treatment with CTX and immunization with anti-id mAb MK2-23 conjugated to KLH and mixed with BCG.

To eliminate the possibility of introducing a lead time-bias into the statistical analysis because of the use of a treatment outcome variable, namely, development of anti-HMW-MAA antibodies, the univariate and multivariate analyses were performed also calculating the survival time from 4 weeks after the first immunization to the end of the study. These analyses were performed only on 46 patients, since 6 did not contribute to positive time. The conclusions of these analyses were identical.
Immunotherapy of Melanoma with Anti-id mAb

...was monitored with 125I-labeled X (2 with PBS-Tween 20. '251-Iabeled antihuman IgG + M washings with PBS-Tween 20, preimmune Cob 38 was incubated for 4 h at 4°C in microtiter plates coated with 0, 7, 28, and 112 with mAb MK2-23 (2 mg/injection) conjugated to KLH. The first two immunizations were given with the immunogen mixed with BCG. A NP40 extract of cultured human melanoma cells Colo 38 was incubated for 4 h at 4°C in microtiter plates coated with anti-HMW-MAA mAbs 149.53 and 763.74 (5 μg/well). Following three washings with PBS-Tween 20, preimmune (II) and immune (II) sera (50 μl/well) were added. Both preimmune and immune sera had been preincubated with an excess of mouse Ig to block antimouse Ig antibodies. Plates were incubated for 4 h at 4°C and then washed three times with PBS-Tween 20. 125I-labeled antihuman IgG + M xenonantibodies (2 x 10^5 cpm/well) were then added, and incubation was continued for an additional 2 h at room temperature. Plates were then washed five times with PBS-Tween 20 and dried. Bound radioactivity was measured in a gamma counter. Results are expressed as bound cpm/well. A NP40 extract of melanoma cells Colo 38 incubated in anti-ICAM-1 mAb CL207.14-coated wells of a microtiter plate was used as a control. The binding of HMW-MAA to mAb 149.53- and to mAb 763.74-coated wells was monitored with 125I-labeled mAb 763.74 (II) and with 125I-labeled mAb 149.53 (II), respectively.

Fig. 3 Reactivity with purified HMW-MAA of serum from patient A. M. immunized with mouse anti-id mAb MK2-23. Serum was obtained on day 182 from patient A. M., who had been immunized on days 0, 7, 28, and 112 with mAb MK2-23 (2 mg/injection) conjugated to KLH. The first two immunizations were given with the immunogen mixed with BCG. A NP40 extract of cultured human melanoma cells Colo 38 was incubated for 4 h at 4°C in microtiter plates coated with anti-HMW-MAA mAbs 149.53 and 763.74 (5 μg/well). Following three washings with PBS-Tween 20, preimmune (II) and immune (II) sera (50 μl/well) were added. Both preimmune and immune sera had been preincubated with an excess of mouse Ig to block antimouse Ig antibodies. Plates were incubated for 4 h at 4°C and then washed three times with PBS-Tween 20. 125I-labeled antihuman IgG + M xenonantibodies (2 x 10^5 cpm/well) were then added, and incubation was continued for an additional 2 h at room temperature. Plates were then washed five times with PBS-Tween 20 and dried. Bound radioactivity was measured in a gamma counter. Results are expressed as bound cpm/well. A NP40 extract of melanoma cells Colo 38 incubated in anti-ICAM-1 mAb CL207.14-coated wells of a microtiter plate was used as a control. The binding of HMW-MAA to mAb 149.53- and to mAb 763.74-coated wells was monitored with 125I-labeled mAb 763.74 (II) and with 125I-labeled mAb 149.53 (II), respectively.

...were entered sequentially into four immunization protocols with the mouse anti-id mAb MK2-23 which bears the internal image of the determinant defined by anti-HMW-MAA mAb 763.74 (3, 5, 17). In more than 30% of the immunized patients the anti-anti-id response was directed to HMW-MAA, since sera from immunized patients displayed a higher reactivity in binding assays with HMW-MAA-bearing melanoma cells than with autologous lymphoid cells which lack HMW-MAA. Furthermore, after absorption with B lymphoid cells, immune sera lost their reactivity with the absorbing cells, but maintained that with melanoma cells. Finally, sera from immunized patients reacted with purified HMW-MAA. This reactivity is mediated by anti-anti-id antibodies, since anti-anti-id antibodies purified from immune sera by affinity chromatography on the immunizing mAb MK2-23 were shown in previous studies (3, 27) to immuno-precipitate HMW-MAA from cultured melanoma cells. The anti-anti-id antibodies in sera from immunized patients are both IgM and IgG. These results, which parallel similar findings in BALB/c mice and rabbits immunized with mAb MK2-23 (4, 5), suggest that the immune response elicited by mAb MK2-23 is T cell dependent. This conclusion is consistent with the ability of mAb MK2-23 to elicit a delayed-type hypersensitivity reaction to HMW-MAA-bearing melanoma cells in BALB/c mice (4).

From a practical point of view, it is noteworthy that the binding assay we have utilized to detect anti-HMW-MAA antibodies in immunized patients offers several advantages. It utilizes a simple and rapid methodology to purify HMW-MAA from cultured melanoma cells, it requires a low amount of serum, it can detect low affinity and/or titer antibodies, and it does not require purification of anti-anti-id antibodies from sera. Therefore, this assay may be suitable to screen large numbers of sera from immunized patients for content of anti-HMW-MAA antibodies.

In agreement with the data obtained in BALB/c mice and rabbits (4, 5), conjugation to a carrier and administration with an adjuvant has markedly enhanced the immunogenicity of anti-id mAb MK2-23 in patients with advanced malignant melanoma, especially as far as induction of anti-HMW-MAA antibodies is concerned. The effect of the carrier is somehow surprising, since one would have expected that the Fc portion of the mouse anti-id mAb would be a carrier for the idiotopes expressed on its variable region. It is noteworthy that mAb MK2-23 conjugated to KLH and mixed with an adjuvant induced anti-HMW-MAA antibodies in more than 90% of the immunized BALB/c mice and rabbits, and in only about 60% of the immunized patients with advanced malignant melanoma. Whether this difference reflects the use of an optimal immunization schedule in the animal model systems but not in patients, lower immunogenicity of anti-id mAb MK2-23 in patients because of its higher resemblance to HMW-MAA expressed by human cells, and/or abnormalities in the immune system of patients because of the disease and/or previous therapy remains to be determined.
Fig. 4  Inhibition of the binding of anti-HMW-MAA mAb 763.74 to cultured human melanoma cells Colo 38 by sera from patients A. M. and T. H. immunized with mouse anti-id mAb MK2-23. Sera were obtained on day 133 from patient A. M. (A) and on day 70 from patient T. H. (B). Patients A. M. and T. H. were immunized on days 0, 7, 28, and 112 and on days 0, 7, 28, 35, and 49, respectively, with mAb MK2-23 (2 mg/injection) conjugated to KLH. The first two immunizations were given with the immunogen mixed with BCG. Melanoma cells Colo 38 were incubated with immune sera of patients for 2 h at 4°C. The supernatant was then carefully removed, and 125I-labeled anti-HMW-MAA mAb 763.74 (0; 2 × 10^7 cpm) was added to each well. Following an additional 2-h incubation at 4°C, cells were washed five times with PBS, and bound radioactivity was measured in a gamma counter. Results are expressed as percentage of inhibition of the binding of 125I-labeled mAb to melanoma cells as compared with binding performed in the presence of preimmune sera. 125I-labeled anti-HMW-MAA mAb 149.53 (○), which defines a spatially distant epitope from that recognized by mAb 763.74, and 125I-labeled anti-ICAM-1 mAb CL207.14 (△) were used as controls.

Fig. 5  Inhibition by anti-id mAb MK2-23 of the binding to cultured human melanoma cells Colo 38 of sera from patients A. M. and T. H. immunized with mouse anti-id mAb MK2-23. Sera were obtained on day 133 from patient A. M. (A) and on day 70 from patient T. H. (B). Patients A. M. and T. H. were immunized on days 0, 7, 28, and 112 and on days 0, 7, 28, 35, and 49, respectively, with mAb MK2-23 (2 mg/injection) conjugated to KLH. The first two immunizations were given with the immunogen mixed with BCG. Immune sera of patients were incubated with anti-id mAb MK2-23 (○) for 2 h at room temperature. The mixture was then added to cultured melanoma cells Colo 38 (2 × 10^5/well), and incubation was continued for an additional 2 h at 4°C. Cells were then washed three times with PBS and incubated with 125I-labeled antihuman IgG + M xenoantibodies (2 × 10^5 cpm/well) for 2 h at 4°C. Following five washings with PBS, bound radioactivity was measured in a gamma counter. Results are expressed as percentage of inhibition of the binding of 125I-labeled antibodies to melanoma cells as compared with binding performed in the presence of mouse IgG. The unrelated anti-id mAb MF11-30 (△) was used as a control.

Low doses of CTX have been reported to enhance cellular and humoral immune responses to tumor vaccines and to potentiate their antitumor effect in both animal model systems and patients (7–10). This effect has been suggested to be caused by a selective inactivation of some subsets of suppressor cells by CTX (11–13). However, we could not detect any effect of CTX on the immunogenicity of anti-id mAb MK2-23 in patients with malignant melanoma. Our results parallel those of Morton et al. (28) and Oratz et al. (29) who did not detect any effect of low doses of CTX on the ability of a polyvalent, allogeneic melanoma vaccine to elicit humoral and/or cellular immunity in patients with malignant melanoma. The discrepancy between the results obtained by Morton et al. (28), Oratz et al. (29), and ourselves and those obtained by other investigators (8–10) may reflect differences in the patient populations treated, timing of CTX administration, and/or differential effect of CTX on the various types of immunogens used.

The development of anti-HMW-MAA antibodies in the immunized patients displayed a statistically significant association with survival prolongation. Given the retrospective nature of the analysis, caution has to be exercised in interpreting this association. Nevertheless, it is not likely to reflect a better
general health condition and function of the immune system of patients who were able to mount a humoral anti-HMW-MAA immune response, since all of the patients were immunocompetent as measured by their ability to produce antiumouse Ig antibodies. Furthermore, the development of humoral anti-HMW-MAA immunity was associated with the survival of patients irrespective of their PS, although the latter variable had an additive effect on this association.

As far as the mechanism(s) underlying the beneficial effect of humoral anti-HMW-MAA immunity on the clinical course of the disease is concerned, we favor the possibility that anti-HMW-MAA antibodies may affect the biology of melanoma cells by inhibiting the function of this molecule. The latter has been suggested to play a role in the metastasizing properties of melanoma cells, since the corresponding monoclonal and polyclonal antibodies inhibit the functional properties of melanoma cells in in vitro assays which correlate with their invasive and metastasizing properties (30–33). Furthermore, in acr lenti- ginous melanoma HMW-MAA has a significantly higher expression in metastatic than in primary lesions (34). In contrast, destruction of melanoma lesions mediated by HMW-MAA binding anti-anti-id antibodies is not likely to play a major role in the clinical course of the disease, since HMW-MAA-binding antibodies from patients immunized with mAb MK2-23 have displayed a poor efficiency in the lysis of melanoma cells in complement and cell-dependent cytotoxicity assays. Furthermore, only three of the immunized patients experienced partial regression of their melanoma lesions following development of anti-HMW-MAA immunity, as described in detail elsewhere (27).

Although the results presented do not formally prove that anti-HMW-MAA immunity plays a causal role in the survival prolongation and in the metastatic lesion regression observed in patients with melanoma immunized with mAb MK2-23, they strongly suggest it. These findings justify the implementation of a double-blind clinical trial to conclusively determine the clinical significance of active, specific immunotherapy with mouse anti-id mAb MK2-23.

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