Syngeneic Anti-idiotypic Antisera to Murine Anti-human High-Molecular Weight Melanoma-associated Antigen Monoclonal Antibodies

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ABSTRACT

BALB/c mice were immunized with the murine antihuman high-molecular-weight melanoma-associated antigen monoclonal antibodies (MoAbs) 149.53, 225.28, 653.25, and 763.74. Anti-idiotypic antibodies could be detected in bleedings obtained 3 days following the first booster, increased in titer in bleedings obtained following the second booster, and persisted at high level in bleedings obtained 38 days following the second booster. Cross-blocking studies with a panel of anti-melanoma-associated antigen, anti-HLA Class I, and anti-HLA Class II monoclonal antibodies showed that the antisera recognize private idiotypes. The latter are located within the antigen combining site, since anti-idiotypic antisera specifically inhibited the binding of the corresponding immunizing anti-human high-molecular-weight melanoma-associated antigen monoclonal antibody to cultured human melanoma cells Colo 38 in a dose-dependent fashion. The spectrotype of the anti-MoAb 149.53 antiserum comprises eight major components in the range of pH 6.2 to 7.0; those of the anti-MoAb 225.28 antiserum and of the anti-MoAb 653.25 antiserum, two major components in the range of pH 6.4 to 6.6 and 6.5 to 6.7, respectively; and that of the anti-MoAb 763.74 antiserum, three major components in the range of pH 6.2 to 6.4.

INTRODUCTION

Among the many human MAA\(^4\) identified with monoclonal antibodies (for review, see Refs. 1 and 2), the HMW-MAA appears to be one of the most useful to develop immunotherapeutic approaches to malignant melanoma. This antigen is membrane bound (3), has a high density on melanoma cells (4), is expressed by a large percentage of primary and metastatic lesions (5), has a restricted distribution in normal tissues (3, 6), displays a limited heterogeneity among both lesions removed from different anatomical sites and tumor cells within a lesion (7), and is modulated only to a limited extent by lymphokines (8, 9). Injection of radiolabeled F(ab\(^{'})\)\(_2\) and Fab\(^{'})\) fragments of anti-HMW-MAA monoclonal antibodies into patients with melanoma has resulted in the specific accumulation of radioactivity in metastatic lesions (10–12). At variance with results reported by Bumol et al. (13), our available anti-HMW-MAA monoclonal antibodies have no effect on the in vitro and in vivo growth of human melanoma cells (14, 15). Even when conjugated to toxins they inhibit only to a limited extent the in vivo growth of human melanoma cells (13, 15). Therefore we are investigating alternative approaches to therapy of melanoma with anti-HMW-MAA monoclonal antibodies. Among them is the application of anti-idiotypic antibodies to anti-HMW-MAA monoclonal antibodies, since injection of rabbit anti-idiotypic antibodies that appear to mimic the structure of p97 MAA into mice has induced immunity to melanoma (16). Furthermore anti-idiotypic antibodies produced when patients were given injections of murine monoclonal antibodies to gastrointestinal tumor cells have been implicated in the clinical improvement and remission of patients with colorectal carcinoma (17).

Since no information is available about the immunogenicity of idiotypes of murine anti-HMW-MAA monoclonal antibodies in syngeneic hosts and since murine anti-HLA Class II monoclonal antibodies differ markedly in their ability to elicit syngeneic anti-idiotypic antibodies (Footnote 5; Ref. 18), as a first step in our immunotherapy program in this study we have developed and characterized syngeneic anti-idiotypic antisera to 4 murine anti-HMW-MAA monoclonal antibodies. The latter include the MoAbs 149.53, 225.28, and 763.74 to distinct determinants of the HMW-MAA (4) and the MoAb 653.25 which reacts with the same or spatially close antigenic determinant as that recognized by the MoAb 225.28 (19).

MATERIALS AND METHODS

Animals. Eight- to 12-wk-old male BALB/c mice were purchased from Charles River Breeding Laboratory, Wilmington, MA.

Cell Lines. Cultured Colo 38 human melanoma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and gentamycin sulfate (25 μg/ml).

Monoclonal Antibodies and Conventional Antisera. The anti-MAA monoclonal antibodies include the MoAbs 149.53, 225.28, 657.5, 763.74, and 902.51 to distinct determinants of the HMW-MAA; the MoAb 225.28 and 653.25 which recognize the same (or spatially close) antigenic determinant on the HMW-MAA (19); the anti-M, 115,000 MAA MoAb 345.134S (20); the anti-A/r 100,000 MAA MoAb 376.96 (21); and the anti-M, 96,000 MAA MoAb CL203.7.4.\(^4\) The anti-HLA Class I monoclonal antibodies include the anti-HLA-A2, A28 MoAb (RII 398.96) (22); the anti-A/r 96,000 MAA MoAb CL203.7.4. The anti-HLA Class I and II monoclonal antibodies include the MoAbs 225.28, 657.5, 763.74, and 902.51 to distinct determinants of the HMW-MAA (4) and the MoAb 653.25 which reacts with the same or spatially close antigenic determinant as that recognized by the MoAb 225.28 (19).

Affinity-purified goat antibodies to the Fc portion of mouse IgG were purchased from Cappel Laboratories (Cochraneville, PA).

Antibodies were labeled with \(^{125}\)I utilizing the iodogen method (29) or the chloramine T method (30). Preparation of F(ab\(^{'})\)\(_2\) Fragments from Monoclonal Antibodies. F(ab\(^{'})\)\(_2\) fragments were generated from monoclonal antibodies following the procedure described by Parham (31). Briefly, purified IgG1 and IgG2a antibodies were incubated with pepsin (Sigma Chemical Co., St. Louis, MO). The enzyme:protein ratios were 1 to 40 and 1 to 8 for IgG1 and for IgG2a monoclonal antibodies, respectively. Digestions were performed at 37°C for 8 h. The pH values of the reaction were 3.5 and 4.1 for IgG1 and IgG2a monoclonal antibodies, respectively. Reactions were stopped by raising the pH to 7.0 with 1 M Tris solution.

\(^{1}\) F. Perosa and S. Ferrone. Syngeneic anti-idiotypic antisera to murine anti-HLA Class II monoclonal antibodies, submitted for publication.


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Then the mixtures were dialyzed against PBS, pH 7.4, and concentrated. Fc fragments and undigested immunoglobulins of IgG2a and IgG1 antibodies were removed by affinity chromatography on Protein A-Sepharose and insolubilized antimurine Fc xenoantibodies, respectively. The purity of F(ab')2 fragment preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed as described by Laemmli (32). The activity of the preparations was assessed by their ability to inhibit the binding of 125I-homologous monoclonal antibodies to melanoma cells.

Production of Syngeneic Antiidiotypic Antisera to Anti-HMW-MAA Monoclonal Antibodies. Four groups of five BALB/c mice each were immunized by injection i.p. of 200 μg of purified MoAbs 149.53, 225.28, 653.25, and 763.74 coupled to solubilized keyhole limpet hemocyanin by glutaraldehyde fixation and mixed with complete Freund’s adjuvant (GIBCO Laboratories, Detroit, MI) (33). Animals were boosted on Days 7 and 14 by an i.p. injection of 200 μg of the same immunogen in incomplete Freund’s adjuvant. Each animal was bled 3 days after the first booster and 3, 10, 24, and 38 days following the second booster.

Idiotype Binding Assay. Polyvinylchloride 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with F(ab’)2 fragments of monoclonal antibodies by incubating 100 μl of a F(ab’)2 fragment solution (100 μg/ml) in 0.1 M bicarbonate buffer, pH 9.5, for 16 h. Following 3 washings with PBS-T20, each well was augmented with 100 μl of antiidiotypic antiserum. Plates were then incubated for 5 h at room temperature. Following 5 washings with PBS-T20, 125I-labeled antihuman Fc xenoantibodies (1 × 105 cpm/ml) were added. At the end of a 4-h incubation, plates were washed 5 times with PBS-T20 and the bound radioactivity was counted in a gamma counter.

Inhibition of Idiotype Binding Assay. Fifty μl of the highest dilution of antiidiotypic antisera given between 60 and 70% of the maximum inhibition to the immunizing monoclonal antibody were incubated for 16 h at 4°C with 50 μl of PBS containing between 1 and 100 μg of monoclonal antibody, used as an inhibitor. Then the mixture was incubated with F(ab')2 fragments of the immunizing anti-HMW-MAA monoclonal antibody with F(ab')2 fragments of an unrelated monoclonal antibody of the same IgG subclass. Results were calculated by subtracting cpm in negative control wells from cpm in experimental wells.

RESULTS

Serial bleedings from BALB/c mice immunized with the anti-HMW-MAA MoAbs 149.53, 225.28, 653.25, and 763.74 reacted with F(ab')2 fragments of the immunizing antibody in the binding assay (Fig. 1). The reactivity of the immune sera with F(ab')2 fragments of monoclonal antibodies is specific, since no binding was detected with preimmune bleedings, and the reactivity of the immune sera was not affected by dilution with normal mouse serum. Antibodies could be detected already in the bleedings obtained 3 days following the first booster. The level of antiidiotypic antibodies, as indicated by the extent of binding and by the titer, increased in the bleedings obtained 3 days following the second booster and persisted unchanged in the serial bleedings obtained up to 38 days following the second booster (Fig. 1).

Fig. 1. Reactivity in the binding assay with the immunizing monoclonal antibody of sera obtained from BALB/c mice 3 days following the first booster (○) and 3 (□), 10 (△), 24 (▲), and 38 (●) days following the second booster with MoAbs 149.53 (A), 225.28 (B), 653.25 (C), and 763.74 (D).
antidiotypic antisera. In binding assays the immune sera did not react with F(ab')2 fragments of any of the anti-HMW-MAA monoclonal antibodies except the immunizing one. In blocking assays the idiotypic-antidiotypic interactions were inhibited neither by anti-MAA monoclonal antibodies, by anti-HLA Class I monoclonal antibodies, nor by anti-HLA Class II monoclonal antibodies (Table 1). Each of the blocking antibodies was used at a concentration 10-fold the minimum amount of homologous antibody required to inhibit idiotypic-antidiotypic reactions by 50%.

To determine the relationship of idiotypes recognized by syngeneic antisera with the antigen combining site, antidiotypic antisera were tested for their ability to inhibit the binding of the immunizing radiolabeled monoclonal antibody to cultured Colo 38 melanoma cells. As shown in Fig. 2, each antidiotypic antiserum inhibited the binding of the homologous 125I-labeled monoclonal antibody to target cells in a dose-dependent fashion. The blocking activity was low in the bleedings obtained 3 days following the first booster, increased in the bleedings obtained 3 days following the second booster, and then persisted unchanged. The titer of the antidiotypic antibodies detected with the blocking assay is similar to that detected with the binding assay with F(ab')2 fragments of monoclonal antibodies. The blocking is specific, since preimmune sera did not affect the binding of 125I-labeled monoclonal antibodies to melanoma cells. Furthermore, each antidiotypic antiserum inhibited only the binding of the immunizing antibody to target cells (Fig. 3).

To analyze the diversity of antidiotypic antibodies, the spectrotype of the antidiotypic antiserum was analyzed by IEF. As shown in Fig. 4, the spectrotype of the anti-MoAb 149.53 antiserum comprises 8 major components in the range of pH 6.2 to 7.0; that of the anti-MoAb 225.28 antiserum, 2 major components in the range of pH 6.4 to 6.6; that of the anti-MoAb 653.25 antiserum, 2 major components in the range of pH 6.5 to 6.7; and that of the anti-MoAb 763.74 antiserum, 3 major components in the range of pH 6.2 to 6.4. No component reacting with the radiolabeled anti-HMW-MAA monoclonal antibodies was detected in the preimmune sera.

**DISCUSSION**

The present study reports for the first time the development and characterization of syngeneic antidiotypic antisera to murine anti-HMW-MAA monoclonal antibodies. A syngeneic system was preferred to a xenogeneic one to minimize the interference of contaminating antibodies in the analysis of the specificity of antidiotypic antibodies and to evaluate the usefulness of immunized BALB/c mice as a source of splenocytes to construct antidiotypic antibodies secreting hybridomas. As it will be reported elsewhere, antidiotypic monoclonal antibodies have been developed utilizing BALB/c mice immunized with the anti-HMW-MAA MoAbs 149.53, 225.28, and 763.74.

The murine immune response is directed to idiotypes located within the antigen combining site of anti-HMW-MAA monoclonal antibodies, since each antidiotypic antiserum inhibited the binding of the immunizing monoclonal antibody to cultured melanoma cells. The presence in the syngeneic antisera of antidiotypic antibodies which do not interfere with antigen-antibody interactions cannot be excluded, especially since analysis of antidiotypic monoclonal antibodies elicited with MoAb 149.53 has identified some which do not inhibit the binding of 125I-labeled immunizing monoclonal antibodies to melanoma.

No significant blocking of the idiotype-antidiotypic reaction with monoclonal antibodies to distinct determinants of the HMW-MAA, to MAA with distinct structural properties, to HLA Class I antigens, and to HLA Class II antigens was detected. As observed in other antigenic systems, not even the anti-HMW-MAA MoAbs 225.28 and 653.25 which appear to recognize the same (or spatially close) antigenic determinant (19) share idiotypes. One might argue that the 15 to 20%
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Fig. 2. Inhibition of binding of I°I-MoAbs 149.53 (A), 225.28 (B), 653.25 (C), and 763.74 (D) to cultured human Colo 38 melanoma cells by homologous antiidiotypic antisera. They were obtained from BALB/c mice 3 days following the first booster (0) and 3 (•), 10 (A), 24 (A), and 38 (•) days following the second booster with MoAbs 149.53 (A), 225.28 (B), 653.25 (C), and 763.74 (D).

Fig. 3. Inhibitory activity of the anti-MoAb 149.53 antiserum (0), anti-MoAb 225.28 antiserum (A), anti-MoAb 653.25 antiserum (Δ), and anti-MoAb 763.74 antiserum (□) on the blocking of I°I-MoAb 149.53 (A), MoAb 225.28 (B), MoAb 653.25 (C), and MoAb 763.74 (D) to cultured human Colo 38 melanoma cells.

anti MoAb 149.53 225.28 653.25 763.74

Fig. 4. Western blot analysis of the binding of I°I-labeled MoAbs 149.53 (Lane A), 225.28 (Lane B), 653.25 (Lane C), and 763.74 (Lane D) to the corresponding syngeneic antiidiotypic antiserum. Isoelectric focusing gels of serum samples were transferred to nitrocellulose, incubated with I°I-labeled carcinoembryonic antibodies, and autoradiographed for between 4 and 12 h.

Inhibition of the idiotype-antiidiotype reaction induced by some of the monoclonal antibodies tested reflects a complete inhibition of one particular minor idiotope binding reaction and therefore detects a shared idiotope. We believe this possibility to be unlikely, since more than 100 antiidiotypic monoclonal antibodies elicited with the anti-HMW-MAA MoAbs 149.53, 225.28, and 763.74 have displayed reactivity only with the immunizing monoclonal antibody. We favor the interpretation that the idiotopes identified with the syngeneic antisera on the 4 anti-HMW-MAA monoclonal antibodies analyzed are private and therefore are likely to be encoded by random somatic mutations in the germ line genes (34). Since the anti-HMW-MAA monoclonal antibodies we have used are secreted by hybridomas generated with splenocytes from mice hyperimmunized with human melanoma cells, it remains to be determined whether cross-reactive idiotopes are expressed by IgM anti-HMW-MAA antibodies, as observed with antiphosphorylcholine and antiphthalate antibodies (35, 36). Furthermore, the range of specificity of antiidiotypic antibodies suggests that the murine antibody repertoire to the human HMW-MAA is broad. This possibility is in agreement with the large number of monoclonal antibodies to distinct determinants of HMW-MAA which have been developed.9

The immunogenicity in syngeneic combinations of the 4 anti-HMW-MAA monoclonal antibodies used, as indicated by the kinetics and titer of antiidiotypic antibodies, is similar. This conclusion parallels similar findings obtained when we have analyzed syngeneic antiidiotypic antisera elicited with 4 monoclonal antibodies to monomorphic and polymorphic determinants of HLA Class I antigens (37). On the other hand, Piatier-Tonneau et al. (18) as well as we have found differences in the titer and in the kinetics of induction of antiidiotypic antibodies elicited with monoclonal antibodies to distinct determinants of HLA Class II antigens.

The immunogenicity in syngeneic combinations of idiotopes of anti-HMW-MAA monoclonal antibodies is similar to that of idiotopes of anti-HLA Class I monoclonal antibodies (37), but higher than that of idiotopes of anti-HLA Class II monoclonal antibodies, as measured by the number of immunizations or by the time required to induce antiidiotypic antibodies. An additional difference between anti-MAA and anti-HLA monoclonal antibodies is represented by the fact that the latter induce not only antibodies to private idiotopes within the antigen combining site, but also antibodies to shared idiotopes which do not interfere with antigen-antibody interactions (Footnote 5; Ref. 37). Whether the latter finding with anti-HMW-MAA and anti-HLA monoclonal antibodies reflects differences in the number of germ line genes or in regulatory controls in the 2 systems remains to be determined.

The spectrotype of the anti-MoAb 149.53 antiserum is broader than that of the anti-MoAb 225.28, anti-MoAb 653.25, and anti-MoAb 763.74 antisera. This finding does not reflect differences in the immunoglobulin class or subclass of the monoclonal antibodies, since like the MoAbs 653.25 and 763.74, the MoAb 149.53 is an IgG1. Furthermore, it does not

9 Unpublished results.
reflect differences in the chemical nature of the determinants recognized by the anti-HMW-MAA monoclonal antibodies, since all of them appear to be carbohydrate in nature.7 Lastly, the similarity in the kinetics of formation and in the reactivity with private idiotopes of the antiidiotypic antisera elicited with the 4 anti-HMW-MAA monoclonal antibodies argues against differences in the immunogenicity of the idiotopes and in the specificity of antiidiotypic antibodies as mechanism(s) underlying the different spectrotypes. Studies in progress with antiidiotypic monoclonal antibodies will determine whether the broad spectrotype of the anti-MoAb 149.53 antisera reflects heterogeneity of antibodies reacting with the same idiotope or of antibodies recognizing distinct idiotopes. The spectrotype of anti-MoAb 225.28, anti-MoAb 653.25, and anti-MoAb 763.74 antisera is similar to that reported for sera from patients immunized with the murine MoAb OKT3 in terms of number of components and of range of isoelectric points (38). These findings suggest that only a limited number of clones are involved in the antibody response to murine monoclonal antibodies in both syngeneic and xenogeneic combinations.

We are aware of only one study which has developed antiidiotypic antisera to murine antihuman MAA monoclonal antibodies. Nepom et al. (16) described rabbit antibodies to idiotopes within the antigen combining site of a murine anti-p97 MAA monoclonal antibody. A component of the antiidiotypic antibodies cross-reacted with 3 of the 4 monoclonal antibodies which recognize the same or spatially close antigenic determinant as the immunizing monoclonal antibody. No cross-reactivity of the antiidiotypic antibodies with monoclonal antibodies which recognize distinct and spatially distant antigenic determinants of the p97 MAA was detected. The difference in the specificity of antiidiotypic antibodies to murine anti-p97 MAA and anti-HMW-MAA monoclonal antibodies may reflect differences in the characteristics of the anti-MAA monoclonal antibodies or in the use of a syngeneic and a xenogeneic system in the 2 studies. If the latter is the case, then this finding parallels similar observations with anti-H-2 monoclonal antibodies but differs from results with anti-HLA Class I monoclonal antibodies. Shared idiotopes of anti-H-2 monoclonal antibodies are recognized by xenogeneic, but not by syngeneic, antiidiotypic antisera (39). On the other hand, syngeneic antiidiotypic antisera to the anti-HLA-A2, A28 MoAb CR11-351 display a reactivity pattern similar to that of xenogeneic antibodies (37, 40).

The potential usefulness to induce immunity to melanoma of antiidiotypic antibodies to private idiotopes within the antigen combining site of anti-HMW-MAA monoclonal antibodies is suggested by recent findings of Kennedy et al. (41) with SV40-transformed cells. These investigators developed rabbit antibodies to private idiotopes within the antigen combining site of murine monoclonal antibodies to SV40 tumor antigen. Injection of antiidiotypic antibodies into BALB/c mice before a tumorigenic dose of SV40-transformed cells suppressed tumor formation. Similar studies have not been performed with murine antiidiotypic antisera to anti-HMW-MAA monoclonal antibodies because of the heterogeneity of the antibody populations in these reagents and of the practical difficulties to prepare large amounts of these reagents. The information resulting from the present investigation has been very useful to plan the strategy to develop syngeneic antiidiotypic monoclonal antibodies to anti-HMW-MAA monoclonal antibodies. Antiidiotypic monoclonal antibodies to the anti-HMW-MAA MoAbs 149.53 and 225.28 have been shown to elicit in BALB/c mice antibodies which can inhibit the binding of the homologous antibodies to human melanoma cells.8 Furthermore, intradermal injection of the antiidiotypic MoAb MF11-30 to the anti-HMW-MAA MoAb 225.28 into 4 patients with melanoma in Stage IV has induced a clinical remission in one of them (41). If these preliminary results are confirmed in a larger number of patients with melanoma, syngeneic antiidiotypic monoclonal antibodies to murine anti-HMW-MAA monoclonal antibodies will represent useful reagents to develop immunotherapeutic approaches to melanoma.

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REFERENCES


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