Introduction

A major challenge of active specific immunotherapy of malignant diseases is the development of effective methods to elicit a strong immune response to human tumor-associated antigens (TAA), most of which are poorly immunogenic or not immunogenic because of their self-nature (reviewed in ref. 1). Several alternative strategies have been tested, including the use of TAA mimics as immunogens. This approach is based on the rationale that (i) B- and T-cell clones that react weakly with self-TAA, unlike those that react strongly, may escape negative selection (e.g., clonal anergy and/or deletion) during the establishment of immunologic self-identity and (ii) these clones may be subsequently stimulated to proliferate by immunization with an immunogen that mimics but is not identical to the original TAA. Several types of TAA mimics have been used. They include xenogeneic TAA (2, 3), which display a high degree of amino acid sequence homology with that of the targeted TAA, peptide mimics (4, 5), and anti-idiotypic antibodies (6–11), both of which display structural homology with the nominal TAA in most cases. Thus far, anti-idiotypic antibodies have been among the most frequently used mimics to implement active specific immunotherapy of malignant diseases. TAA-specific immunity elicited by anti-idiotypic antibodies has been shown to exert beneficial effects on the clinical course of several malignant diseases (6–12).

We have used anti-idiotypic monoclonal antibodies (mAb) to implement active specific immunotherapy of melanoma targeting the human high molecular weight melanoma-associated antigen (HMW-MAA). Like other self-TAA, HMW-MAA is not immunogenic in hosts with constitutive expression of this antigen (13). To overcome unresponsiveness to this antigen, we have immunized melanoma patients with the anti-idiotypic mAb MF11-30 and MK2-23, which mimic the antigenic determinants defined by anti-HMW-MAA mAb 225.28 and 763.74, respectively (6, 7). In agreement with the results obtained in preclinical models (14), we have showed that conjugation of mAb MK2-23 with keyhole limpet hemocyanin (KLH) and coadministration with Bacillus Calmette-Guerin (BCG) as an adjuvant is required to elicit HMW-MAA-specific anti-anti-idiotypic antibodies in melanoma patients. Although their induction was associated with survival prolongation (7, 8), the clinical use of this strategy has been hampered by difficulties in standardizing KLH-mAb conjugates and by side effects associated with BCG administration, such as erythema and ulceration at the injection sites (7, 8). These limitations have stimulated interest in the development of alternative immunization strategies that are effective in enhancing the immunogenicity of mAb MK2-23 but do not require the use of KLH and BCG. Therefore, in the present study, we have tested whether fusion to the immunostimulatory cytokine interleukin 2 (IL-2) enhances the immunogenicity of mAb MK2-23.

Materials and Methods

Cell lines and hybridomas. Cultured human SK-MEL-28 melanoma cells, human LG2 B-lymphoid cells, mouse Sp2/0-Ag14 myeloma cells, IL-2-dependent mouse CTLL-2 CTL cells, mouse hybridoma cells secreting...
mouse mAb MK2-23 and mouse hybridoma cells secreting chimeric mAb MK2-23 (chMK2-23) were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Sigma, St. Louis, MO) in a humidified 5% CO2 atmosphere.

**Monoclonal and polyclonal antibodies.** The HMW-MAA-specific mAb 763.74 (15); HLA-A2, HLA-A24, HLA-A28-specific mAb CR11-351 (16); CD4-specific mAb GK1.5 (17); CD8-specific mAb 2.43 (18); anti-idiotypic mAb MK2-23, which mimics the determinant recognized by mAb 763.74 (19); and anti-idiotypic mAb T3-203, which mimics the determinant recognized by mAb CR11-351 (20), were developed and characterized as described. mAbs were purified from ascitic fluid by sequential ammonium sulfate and caprylic acid precipitation (21). F(ab')2, fragments were obtained by pepsin digestion of mouse mAb as previously described (19); their purity and activity were monitored by SDS-PAGE and by testing with the corresponding antigen in ELISA, respectively. Affinity-purified goat anti-mouse IgG (Fc specific) antibodies, affinity-purified goat anti-mouse IgM (μ chain specific) antibodies and affinity-purified, horseradish peroxidase (HRP)–conjugated rabbit anti-human IgG (Fc specific) antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), FITC-conjugated CD4-specific mAb GK1.5 and CD8-specific mAb 2.43 were purchased from BD PharMingen (San Jose, CA).

Antibodies were radiolabeled with Na125I (Amersham, Piscataway, NJ) by the iodogen method (22), using IODO-GEN Precoated Reaction Tubes (Pierce, Rockford, IL). mAbs were pooled into parallel aliquots (20 μl) and stored at −80°C for future use. mAb MK2-23 VH and VL chains were synthesized in a DNA synthesizer (BioSearch; MilliGen/Biosearch, Bedford, MA). Primers were designed to provide amplification of mAb MK2-23 heavy (VH) and light (VL) chain variable regions that were synthesized in a DNA synthesizer (BioSearch; MilliGen/Biosearch, Bedford, MA). Primers included a restriction enzyme (restriction site), 5'-GACTTGCTCAGCTCAGTACGGTGCTC-3' (VH forward primer) and underlined portion denotes XhoI restriction site), 5'-CTAGGTCTACCT-GAGGACGCTTACGGTGG-3' (VL reverse primer; underlined portion denotes HindIII restriction site), 5'-GAGTCTAGAGATGAGGACAAGACA- CACTCAGGGTTACTCATCCGTGGTCC-3' (VH forward primer; underlined portion denotes BstHI restriction site), 5'-GTCGATCTCCACGATTTTTTGACCCACCTGCACGGTGG-3' (VL reverse primer; underlined portion denotes BamHI restriction site).

**Recombinant human interleukin 2.** Lyophilized, recombinant human IL-2 (Proluken/Aldesleukin; specific activity: 3 × 10^8 Cetus units/mg Chiron Corp., Emeryville, CA) was reconstituted in sterile Milli-Q water to a concentration of 200 μg/mL, aliquoted, and stored at −80°C until use. Directly before injection, this stock solution was further diluted to the working concentration in 5% dextrose solution.

**Animals.** Six- to 8-week-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA).

**Construction and purification of MK2-23-IL-2 fusion protein and chimeric mAb MK2-23 (chMK2-23).** mAb MK2-23 VH and VL chains were amplified from polyadenyllic acid (poly(A)+) RNA obtained from hybridoma cells secreting mAb MK2-23 by reverse transcriptase-PCR, as described (24). The PCR products were cloned into the pCR-Script SK(+) vector (Stratagene, La Jolla, CA) with the pCR-Script SK(+) cloning kit (Stratagene) for sequencing. After mAb MK2-23 VH and VL chain sequences were verified, mAb MK2-23 VH and VL chain cDNA were subcloned into the vector pDHIL2, which contains different transcriptional units coding for (i) the constant region of human Ig γ1 heavy chain, fused at the carboxyl end to a synthetic sequence coding for human IL-2 as described (25); (ii) the constant region of human Ig κ light chain; and (iii) a mutant dihydrofolate reductase gene, as described (26, 27). Briefly, an XhoI-HindIII fragment containing mAb MK2-23 VH chain cDNA was joined to the 5' end of the κ light heavy chain constant region. Likewise, an XhoI-BamHI fragment containing mAb MK2-23 VH chain cDNA was joined to the 5' end of the κ light chain constant region. chMK2-23 was constructed as described for the MK2-23-IL-2 fusion protein, except that the pDHIL2 vector contained the coding sequence for the human IgG1 constant region followed by its native 3' untranslated region and poly(A) adenylation site rather than the human IL-2 sequence. The pHIL2 plasmid was then introduced into mouse Sp2/0-Ag14 myeloma cells by protoplast fusion and successfully transfected myeloma cells were selected in DMEM supplemented with 10% FCS and 100 mmol/L methotrexate. Selected cells were subsequently grown in hybridoma serum-free medium (Life Technologies) supplemented with 5% FCS and 100 mmol/L methotrexate. MK2-23-IL-2 fusion protein and chMK2-23 chimeric mAb were purified from spent culture medium of transfected Sp2/0-Ag14 myeloma cells by affinity chromatography on mAb 763.74 coupled to NHS-activated Sepharose and on Protein A-Sepharose (Repligen, Waltham, MA), respectively. The purity of the fusion protein and chimeric mAb were analyzed by 10% SDS-PAGE and staining with Coomassie blue.

**Assessment of IL-2 activity of MK2-23-IL-2 fusion protein.** The IL-2 activity of the MK2-23-IL-2 fusion protein was assayed as described (25). Briefly, a standard T-cell proliferation assay was done using mouse CTLL-2 cells grown in DMEM supplemented with 10% FCS which had been IL-2-depleted for 48 hours before the assay.

**Immunization schedule.** Five mice were immunized s.c. on days 0, 28, and 84 with MK2-23-IL-2 fusion protein (100 μg/injection; group 1). As controls, five mice were immunized with mAb MK2-23 (group 2), five with chMK2-23 (group 3), five with KLH-conjugated MK2-23-IL-2 fusion protein (group 4), and five with KLH-conjugated mAb MK2-23 (group 5), using the same route, schedule and dose. Additionally, mice in groups 2, 3, and 5 were injected with IL-2 s.c. twice daily for 5 days (0.1 μg/g body weight/injection), starting on the day of each immunization, as described (28). Sera were harvested from each group of mice 1 day before the first immunization and at weekly intervals after the start of immunization.

**Binding assays.** The RIA to detect anti–anti-idiotypic antibodies in mouse sera that react with mAb MK2-23 F(ab')2 fragments and with melanoma cells was done in 96-well flexible microtiter plates (Costar, Corning, Acton, MA) with 125I-labeled anti-mouse IgG, as described (14, 19). Briefly, microtiter plates were coated with antibodies [1 μg per well] in 0.1 mol/L NaHCO3 (pH 9.6) O/N at room temperature, and were then blocked with 2% bovine serum albumin (BSA)/PBS for 2 hours at room temperature. Two-fold dilutions of sera were added, and the mixture was incubated for 2 hours at room temperature. Following three washings with 0.05% Tween 20/PBS and two washings with PBS, 125I-labeled anti-mouse IgG xenoantibodies (1 × 10^6 cpm per well) were added to each well and incubation was continued for one additional hour at room temperature. Plates were then washed thrice with 0.05% Tween 20/PBS and twice with PBS. Wells were then cut, and bound radioactivity was counted in a gamma counter (Packard Cobra II Auto Gamma Model 5002, Perkin-Elmer, Downers Grove, IL). Results are expressed either as cpm or as titer (defined as the highest dilution of sera which results in 50% of the maximal binding to the antigen). The binding assay with cells was done following the same procedure, except that (i) all incubation steps were done at 4°C, (ii) a solution of 0.5% BSA/PBS was used for wash steps, and (iii) cells were centrifuged at 750 × g for 5 minutes following each wash step.

A solid-phase ELISA to determine the IgM and IgG class of anti–anti-idiotypic antibodies in mouse immune sera was done in 96-well flexible microtiter plates (Costar), which were coated with antibody (1 μg per well) O/N at room temperature. The wells were then blocked with 2% BSA/PBS for 2 hours at room temperature. After washing, serially diluted mouse sera were added to the wells, and the plate was incubated for 2 hours at room temperature. Bound antibodies were detected by sequential incubation with HRP-conjugated goat anti-mouse IgM or IgG antibodies for 1 hour at room temperature and with orthophenylenediamine (Sigma) for 5 minutes at room temperature. Results are expressed as absorbance, which was determined at 490 nm on an ELISA plate reader (Bio-Tek EL311, Winooski, VT). The binding assay to determine the reactivity of the MK2-23-IL-2 fusion protein was done in a similar manner, except that (i) biotinylated mAb were used as the probe; (ii) bound biotinylated mAb were detected by sequential incubation with HRP-streptavidin for 1 hour at room temperature; and with 3,3',5,5'-tetramethylbenzidine (TrueBlue Peroxidase Substrate, KPL, Gaithersburg, MD) for 5 minutes at room temperature; and (iii) absorbance was determined at 450 nm.

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Flow cytometry analysis. Flow cytometry analysis of cells was done as described (29). Briefly, 5 x 10^6 cells were incubated for 1 hour at 4°C with fluorochrome-conjugated mAb recognizing the appropriate surface antigen. Cells were then washed twice with 0.5% BSA/PBS, fixed in 2% paraformaldehyde and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA). Results were analyzed with CellQuest software and were expressed as fluorescence intensity units.

Immunofluorescence localization. Labeling of cells with 125I, solubilization with 1% NP40, indirect immunoprecipitation, SDS-PAGE, and autoradiography were done as described (19).

Delayed-type hypersensitivity assay. Immune mice were injected s.c. in the footpad on day 93 with SK-MEL-28 or LG2 cells (5 x 10^6 cells per mouse), which had been irradiated with 10,000 rad and resuspended in 20 μl HBSS. Footpad thickness at 0, 24, 48, and 72 hours after cell injection was determined using calipers. The swelling induced by injection of cells was determined by subtracting the footpad thickness at 0 hour from that after the injection of cells at the indicated time points.

In vivo cell depletion. To deplete CD4+ and/or CD8+ cells in vivo, mice were injected i.v. with CD4-specific mAb GK1.5 and/or CD8-specific mAb 2.43 (500 μg per mouse per mAb). Flow cytometry analysis of splenocytes from mice after injection with anti-CD4 and/or anti-CD8 mAb consistently revealed >95% depletion of the corresponding cell subset, which was observed at 24 hours after mAb injection and persisted for up to 7 days (data not shown).

Statistical analysis. The statistical significance of the difference in specific antibody levels and in the delayed-type hypersensitivity (DTH) response among groups of mice was analyzed using the two-tailed, unpaired Student’s t test. A difference was considered statistically significant when the P < 0.05.

Results

Characterization of MK2-23-IL-2 fusion protein. The MK2-23-IL-2 fusion protein was purified from spent culture medium of transfected myeloma cells by immunofluorescence chromatography on insolubilized mAb 763.74. By SDS-PAGE analysis under nonreducing conditions, the MK2-23-IL-2 fusion protein migrated to a position corresponding to a slightly higher apparent molecular weight than that of the parental mAb MK2-23. Under reducing conditions, the MK2-23-IL-2 fusion protein resolved into two distinct components at ~70 and ~30 kDa, which correspond to the human IgG heavy chain fused to IL-2 and the human IgG light chain, respectively. In contrast, under reducing conditions, parental mAb MK2-23 resolved into two distinct components at ~55 and ~28 kDa, which correspond to the mouse IgG heavy and light chains, respectively (Fig. 1). The difference in the apparent molecular weight of the IgG heavy chains of the MK2-23-IL-2 fusion protein and of the parental mAb MK2-23 corresponds approximately to the molecular weight of glycosylated IL-2 (15-18 kDa; ref. 30). This finding is compatible with the covalent linkage of IL-2 to the human IgG heavy chain in the fusion protein. Additionally, the small difference in the apparent molecular weight between the MK2-23-IL-2 fusion protein light chain and the parental mAb MK2-23 light chain may correspond to differences in the amino acid sequences between the mouse and human constant regions, which result in their slightly different charges and electrophoretic mobility.

The MK2-23-IL-2 fusion protein stimulated proliferation of mouse CTLL-2 cells in IL-2-depleted medium, indicating that IL-2 expressed in the fusion protein is functionally active. The concentration of fusion protein required to stimulate 50% of the maximum [3H]-thymidine incorporation (i.e., ED50) is ~1 ng/mL (Fig. 2A), which corresponds to a specific IL-2 activity of 1 x 10^8 units/mg. Based on the ~16% weight ratio of IL-2 in the fusion protein, the specific activity for the IL-2 component of the fusion protein is 6 x 10^8 units/mg (ED50 = 0.16 ng/mL). This level of bioactivity is comparable with that of commercially available recombinant human IL-2 (ED50 = 0.05-0.25 ng/mL; available from R&D Systems, Minneapolis, MN). Additionally, the MK2-23-IL-2 fusion protein reacted with biotinylated mAb 763.74 at a similar level as the parental mAb MK2-23 (Fig. 2B). In both cases, the reactivity was dose dependent.

Immunogenicity of MK2-23-IL-2 fusion protein in immunized mice. Mice were immunized with the MK2-23-IL-2 fusion protein on days 0, 28, and 84. Anti-anti-idiotypic and anti-HMW-MAA antibodies, as measured by their reactivity with F(ab')2 fragments of mAb MK2-23 and with HMW-MAA+ SK-MEL-28 cells, respectively, were detected in the sera harvested 7 and 14 days after priming (Fig. 3A; data not shown). The level of anti-anti-idiotypic IgG antibodies was markedly higher than that of anti-anti-idiotypic IgM antibodies in the sera harvested 7 days post-priming and increased in sera harvested 14 days post-priming (Fig. 3D). These findings indicate that the MK2-23-IL-2 fusion protein can elicit antibodies that undergo rapid class switching. Sera obtained from mice 7 days after the third immunization showed a high level of reactivity with mAb MK2-23 F(ab')2 fragments (Fig. 3B) and with HMW-MAA+ SK-MEL-28 melanoma cells (Fig. 4). These sera immunoprecipitated components exhibiting the characteristic electrophoretic profile of HMW-MAA from 125I-labeled HMW-MAA+ SK-MEL-28 cell lysate (Fig. 5).

The mice immunized with MK2-23-IL-2 fusion protein showed a HMW-MAA-specific DTH response, because they exhibited significantly (P < 0.005 for all comparisons) higher footpad swelling in response to injection of HMW-MAA+ SK-MEL-28 cells than HMW-MAA− LG2 cells (Fig. 6A). Marked swelling was observed 24 hours after injection and persisted up to 72 hours. The HMW-MAA-specific DTH reaction was both CD4+ and CD8+ cell dependent, because the mice immunized with MK2-23-IL-2...
fusion protein but depleted of either CD4+ or CD8+ cells exhibited a partial loss of HMW-MAA-specific DTH reactivity (Fig. 6B). Interestingly, the depletion of CD8+ cells seemed to abrogate the HMW-MAA-specific DTH response only at the 24-hour time point, whereas the depletion of CD4+ cells seemed to abrogate the specific DTH response only at the 72-hour time point. In contrast, the mice immunized with MK2-23-IL-2 fusion protein but depleted of both CD4+ and CD8+ cells exhibited a loss of HMW-MAA-specific DTH response at all of the time points tested (24, 48, and 72 hours after cell injection; Fig. 6B).

Requirement of conjugation to monoclonal antibody MK2-23 for the adjuvant role of interleukin 2. An additional group of mice was immunized with mAb MK2-23 by the same immunization schedule as for the MK2-23-IL-2 fusion protein and was given IL-2 by an adjuvant schedule as described (28). Anti–anti-idiotypic antibodies were detected in immune serum harvested 14 days post-priming (data not shown). Immune sera harvested 7 days after the third immunization from the mice immunized with mAb MK2-23 and IL-2 revealed significantly lower reactivity with immobilized mAb MK2-23 F(ab')2 fragments (P < 0.001; Fig. 3B) and with HMW-MAA+ SK-MEL-28 cells (P < 0.05; Fig. 4B) than sera obtained from the mice immunized with MK2-23-IL-2 fusion protein at the same time point. This finding is consistent with the observation that sera from these mice were unable to immunoprecipitate proteins with the characteristic electrophoretic profile of HMW-MAA from 125I-labeled SK-MEL-28 cell lysate (data not shown). Furthermore, in contrast to the pronounced DTH response observed in the mice immunized with MK2-23-IL-2 fusion protein, mAb MK2-23 by itself seemed to abrogate the HMW-MAA-specific DTH response only at the 24-hour time point. The mice immunized with mAb MK2-23 and IL-2 seemed to abrogate the specific DTH response only at the 72-hour time point. In contrast, the mice immunized with MK2-23-IL-2 fusion protein but depleted of both CD4+ and CD8+ cells exhibited a loss of HMW-MAA-specific DTH response at all of the time points tested (24, 48, and 72 hours after cell injection; Fig. 6B).

Figure 2. IL-2 activity and antigenicity of MK2-23-IL-2 fusion protein. A, IL-2 activity of MK2-23-IL-2 fusion protein was measured by [3H]-thymidine incorporation by mouse CTLL-2 cells in IL-2-depleted medium. The concentration of fusion protein required to stimulate 50% of the maximum [3H]-thymidine incorporation (dashed horizontal line) is 1 ng/mL, indicating a specific activity of 1 × 10^6 units/mg fusion protein, or 6 × 10^6 units/mg IL-2 based on the weight ratio of IL-2 in the fusion protein. B, serially diluted MK2-23-IL-2 fusion protein, parental mAb MK2-23 (anti-idiotypic mAb of mAb 763.74) or irrelevant mAb T8-203 (anti-idiotypic mAb of mAb CR11-351) was coated on a microtiter plate. The fusion protein or mAb was then incubated with biotinylated mAb 763.74 (0.175 μg per well, ●). Biotinylated mAb CR11-351 (0.175 μg per well, ○) was used as a specificity control. Bound biotinylated mAb was detected by sequential incubation with HRP-SA and with TMB.

Figure 3. Level of anti–anti-idiotypic antibodies in sera from mice immunized with MK2-23-IL-2 fusion protein. A, mice (n = 5 per group) were immunized on day 0 with MK2-23-IL-2 fusion protein and sera were harvested 1 and 2 weeks after priming. Anti–anti-idiotypic antibodies of the IgM subclass in 1-week sera (top) and of the IgG subclass in 1-week (middle) and 2-week (bottom) sera, were detected by ELISA with mAb MK2-23 F(ab')2 fragments (1 μg per well). Bound antibodies were detected by sequential incubation with HRP-conjugated anti-mouse IgM or IgG antibodies and with orthophenylenediamine. B, mice (n = 5 per group) were immunized on days 0, 28, and 84 with MK2-23-IL-2 fusion protein, mAb MK2-23 + IL-2, chMK2-23 + IL-2, KLH-MK2-23-IL-2, or KLH-MK2-23 + IL-2, and sera were harvested 1 week after the last immunization. Anti–anti-idiotypic antibodies were detected by RIA with 125I-labeled anti-mouse IgG xenoantibodies, and were quantitated as cpm using a gamma counter. Columns, means of the sera dilution giving 50% of the maximal binding to mAb MK2-23; bars, ± SD. ***, P < 0.001 versus mAb MK2-23 + IL-2; ##, P < 0.01 versus chMK2-23 + IL-2.
fusion protein, the mice immunized with mAb MK2-23 and IL-2 did not show a HMW-MAA-specific DTH response ($P > 0.05$ for all comparisons; Fig. 6A). Therefore, IL-2 must be conjugated to mAb MK2-23 to enhance the HMW-MAA-specific immune responses elicited by mAb MK2-23.

Immunization with chMK2-23, in combination with IL-2, did not generate a significantly greater HMW-MAA-specific antibody response than immunization with the parental mAb MK2-23 and IL-2 (Fig. 4). Furthermore, immunization with chMK2-23 and IL-2 produced a significantly lower anti–anti-idiotypic ($P < 0.01$) and anti-HMW-MAA ($P < 0.05$) antibody response than immunization with MK2-23-IL-2 fusion protein. Therefore, the increased immunogenicity of the MK2-23-IL-2 fusion protein is due to the ability of conjugated IL-2 to mediate an adjuvant effect, rather than to the ability of the human IgG constant regions to increase the immunogenicity of mAb MK2-23 variable regions.

Lack of detectable effect of keyhole limpet hemocyanin conjugation on the immunogenicity of MK2-23-IL-2 fusion protein. Additional groups of mice were immunized with KLH-conjugated MK2-23-IL-2 fusion protein (subsequently called "KLH-MK2-23-IL-2"), or with KLH-conjugated mAb MK2-23 (subsequently called "KLH-MK2-23") in combination with IL-2. The titer of anti–anti-idiotypic antibodies in sera from the mice immunized with KLH-MK2-23-IL-2 was comparable to that in sera from the mice immunized with MK2-23-IL-2 fusion protein but was significantly higher ($P < 0.001$) than that in sera from the mice immunized with mAb MK2-23 in combination with IL-2 (Fig. 3B). The average titer of anti–anti-idiotypic antibodies in sera from the mice immunized with KLH-MK2-23 and IL-2 was higher than that in sera from the mice immunized with mAb MK2-23 and IL-2; however, this difference was not statistically significant ($P > 0.05$; Fig. 3B).

The titer of HMW-MAA-specific antibodies in sera from the mice immunized with KLH-MK2-23-IL-2 was significantly higher ($P < 0.05$) than that in sera from the mice immunized with KLH-MK2-23 and IL-2 (Fig. 4B). However, conjugation of the fusion protein with KLH did not seem to enhance the titer of HMW-MAA-specific reactivity than sera from the mice immunized with mAb MK2-23 and IL-2, sera from the mice immunized with KLH-MK2-23 and IL-2 exhibited a ~2-fold higher HMW-MAA-specific reactivity than sera from the mice immunized with MK2-23-IL-2.
Discussion

We have shown that the MK2-23-IL-2 fusion protein can elicit rapid, robust anti–anti-idiotypic and anti-HMW-MAA antibody responses. In addition, immunization with the MK2-23-IL-2 fusion protein generated HMW-MAA-specific cellular responses that were CD4+ and CD8+ cell dependent. Both the humoral and cellular immune responses elicited by immunization with MK2-23-IL-2 fusion protein were significantly higher than those elicited by immunization with mAb MK2-23 and administration of IL-2, indicating that conjugation of IL-2 to mAb MK2-23 is critical to the immunogenicity of the fusion protein. The enhanced immunogenicity of the fusion protein cannot be attributed to the human IgG constant region, because immunization with chMK2-23 does not significantly enhance HMW-MAA-specific antibody responses compared with immunization with the parental mouse mAb MK2-23. The immunogenicity of the MK2-23-IL-2 fusion protein was not increased by conjugation to KLH.

A comparison of the immunogenicity of the MK2-23-IL-2 fusion protein with that of KLH-conjugated mAb MK2-23 coadministered with Freund’s adjuvant (14) showed that the immune responses in BALB/c mice were similar, as measured by the levels of HMW-MAA-specific cellular and humoral immune responses. These findings suggest that the MK2-23-IL-2 fusion protein can be used as an effective immunogen without the need for carriers or adjuvants. In the clinical setting, such a strategy avoids the use of KLH as a carrier and BCG as an adjuvant, thus bypassing the limitations associated with the clinical application of these two agents (7, 31).

The HMW-MAA-specific DTH response elicited by MK2-23-IL-2 fusion protein suggests that HMW-MAA-specific T cells have been generated by this immunogen. This specific DTH response was abrogated if either CD4+ or CD8+ cells were depleted before injection of HMW-MAA-bearing cells. In addition, depletion of both CD4+ and CD8+ cells resulted in a greater loss of HMW-MAA-specific DTH reactivity than depletion of either T-cell subset alone. Collectively, these findings suggest that the MK2-23-IL-2 fusion protein can elicit both HMW-MAA-specific CD4+ and CD8+ T cells.

Our findings are consistent with those from several recent reports, which indicate that anti-idiotypic mAb which mimic TAA can elicit TAA-specific CTL responses in animal model systems (32, 33) and in patients with malignant diseases (12, 34). Furthermore, the presence of anti–anti-idiotypic and anti-HMW-MAA IgG antibodies in the serum of mice immunized with the MK2-23-IL-2 fusion protein suggests that the HMW-MAA-specific CD4+ T cells may also mediate other immunologic roles, such as immunoglobulin class switching. Interestingly, our depletion experiments indicated that the DTH response was mediated by CD8+ T cells at earlier time.

Figure 6. DTH reaction to SK-MEL-28 melanoma cells (■) or LG2 lymphoid cells (□) in mice immunized with MK2-23-IL-2 fusion protein. A, mice (n = 5 per group) were immunized on days 0, 28, and 84 with MK2-23-IL-2 fusion protein, mAb MK2-23 + IL-2, KLH-MK2-23-IL-2, or KLH-MK2-23 + IL-2, and were injected in the footpad with 5 × 10^5 cells in HBSS on day 93. Footpad thickness at 24, 48, and 72 hours after cell injection were determined with calipers and compared with the corresponding thickness at 0 hour. B, mice were immunized with MK2-23-IL-2 fusion protein, as described in (A). Twenty-four hours before footpad injection with 5 × 10^5 cells in HBSS on day 93, the mice (n = 5 per group) were injected with anti-CD4 and/or anti-CD8 mAb (500 μg per mouse per mAb) to deplete CD4+ and/or CD8+ cells, respectively. An additional group of immunized mice was not injected with either anti-CD4 or anti-CD8 mAb, to serve as a non–cell-depleted immunization control. The DTH reaction was monitored as in (A). Columns, means; bars, ±SD. *, P < 0.05; **, P < 0.005; ***, P < 0.001 for the indicated comparisons.
points (e.g., 24 hours) and by CD4+ T cells at later time points (e.g., 72 h). These observations mirror those from a previous report, which showed a biphasic T cell–mediated lymphocytic chorionemangitis virus–specific DTH response that was mediated by CD8+ T cells in an earlier phase and by CD4+ T cells in a later phase (35).

Several potential mechanisms, which are not mutually exclusive, may explain how fusion of an anti-idiotype mAb with IL-2 enhances its immunogenicity. First, interaction of the Fc component of the fusion protein with Fc receptors expressed on antigen-presenting cells (APC), such as dendritic cells and macrophages, may localize IL-2 to the APCs. This would stimulate the APCs, leading to an increase in proliferation, maturation, antigen presentation, and/or secretion of cytokines. These changes may increase the ability of APCs to prime antigen-specific immune effector cells. In addition, the migration of these cells to secondary lymphoid organs (e.g., lymph nodes) may lead to greater localization of the fusion protein at these antigen presentation sites, thereby increasing the presentation of the anti-idiotype mAb and stimulation of effector cells. Second, the fusion protein may bind to APCs or anti-idiotype mAb-specific T-cell subsets because these cells express IL-2 receptors on their surface (36). As a consequence, the fusion protein can localize to sites where these cells are abundant, such as lymph nodes, and can also trigger IL-2-dependent maturation, activation, and proliferation of these cells.

Third, the anti-idiotype mAb component of the fusion protein may trigger an idiotype cascade *in vivo* (37). The idiotype cascade may lead to formation of immune complexes that are phagocytosed and processed by APCs, leading to a greater level of antigen presentation and an increase in antigen-specific antibody and cellular responses that is further enhanced by the presence of IL-2. Fourth, the fusion protein may prolong the half-life of each of its components by making them less susceptible to degradation *in vivo*. Prolongation of the half-life of IL-2 and granulocyte-macrophage colony-stimulating factor has been reported when these cytokines are fused to antibodies (38).

We have selected IL-2 for our fusion protein, because IL-2 has been shown to exert pleiotropic roles in the modulation of antigen-specific immune responses. The central role of IL-2 as an adjuvant is based primarily on its ability to trigger the proliferation and differentiation of antigen-specific lymphocytes, particularly CD4+ T helper cells (39) but also including B cells (39, 40) and CD8+ T cells (41). Our findings parallel those of other investigators who have used anti-idiotype antibodies conjugated to immunostimulatory cytokines as immunogens. In a recent study, the conjugation of IL-6 to anti-idiotype single-chain Fv antibody ACA125, which mimics the CA125 ovarian carcinoma antigen, enhanced the titer of antibodies that are reactive with the parental anti-idiotype mAb and with CA125+ ovarian carcinoma cells when compared with the administration of the ACA125 and IL-6 components separately (42).

However, this fusion protein was given with Freund's adjuvant, and it is not known if the fusion protein is more immunogenic than the parental mAb ACA125 and IL-6 as well as if Freund's adjuvant is required to induce an anti-anti-idiotype response. Furthermore, it is not known if this fusion protein can elicit a cellular immune response to the nominal antigen.

It is noteworthy that the HMW-MAA has conserved its structural and functional properties through phylogenetic evolution. Mice express a homologue of HMW-MAA termed AN2. This molecule shares >80% amino acid sequence homology with the human HMW-MAA (43). Because the MK2-23-IL-2 fusion protein was able to elicit HMW-MAA–specific antibody and cellular responses in a host with a constitutive expression of a highly homologous antigen, a similar strategy may be effective in breaking unresponsiveness to self-HMW-MAA in patients with melanoma. This possibility is supported by the close correlation we have previously found between the immune response elicited by mouse anti-idiotype mAb MK2-23 in BALB/c mice (14) and in patients with melanoma (6, 7), and in the variables which regulate this immune response.

In conclusion, fusion with IL-2 enhances the immunogenicity of mAb MK2-23 and bypasses the requirement for conjugation to a carrier and administration with an adjuvant. Immunization with the MK2-23-IL-2 fusion protein may represent an effective therapeutic strategy in patients with HMW-MAA-bearing tumors, such as melanoma.

**Acknowledgments**

Received 6/30/2004; revised 4/30/2005; accepted 5/5/2005.

**Grant support:** National Cancer Institute/USPHS grants P01 CA89480 and B01 CA105500, Department of Health and Human Services, and Harry J. Lloyd Charitable Trust.

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**References**


Human High Molecular Weight Melanoma-Associated Antigen Mimicry by Mouse Anti-Idiotypic Monoclonal Antibody MK2-23: Enhancement of Immunogenicity of Anti-Idiotypic Monoclonal Antibody MK2-23 by Fusion with Interleukin 2


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