Interleukin-6 Fused to an Anti-idiotype Antibody in a Vaccine Increases the Specific Humoral Immune Response against CA125\(^+\) (MUC-16) Ovarian Cancer\(^1\)

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ABSTRACT

Anti-idiotype (Id) monoclonal antibodies can serve as surrogate for tumor-associated antigens in vaccination strategies. The murine anti-Id monoclonal antibody ACA125 that mimics the CA125 carbohydrate antigen expressed on ovarian cancer cells can serve an anti-anti-Id antibody (Ab3) response that is associated with prolonged survival of ovarian cancer patients. To increase the Ab3 antibody response, we evaluated two strategies in a mouse model: (a) coinjection of human interleukin (IL)-6 together with the fusion protein chACA125, which consists of the anti-Id ACA125 single-chain Fv antibody joined to the human IgG1 CH2/CH3 domain; (b) injection of the fusion protein chACA125-IL-6, which consists of the ACA125 single-chain Fv fused to human IL-6 via the IgG1 CH2/CH3 domain. Vaccination of mice with the chACA125-IL-6 fusion protein resulted in higher titers of anti-CA125 (Ab3) antibodies compared with application of the chACA125 antibody with or without systemic coadministration of IL-6. Application of the chACA125-IL-6 fusion protein did not elicit detectable antihuman IL-6 antibody titers, whereas coinjection of human IL-6 did. Taken together, these data suggest that the chACA125-IL-6 fusion protein directly stimulates ACA125-specific B cells via the IL-6 domain, whereas coinjection of IL-6 leads to an overall immune stimulation. Antigen-IL-6 fusion proteins will improve vaccination regimens and anticancer immunotherapeutic strategies by increasing the antigen-specific humoral immune response.

INTRODUCTION

Anti-Id\(^1\) antibodies (Ab2) can successfully serve as a surrogate for tumor antigen in vaccination strategies by triggering humoral and cellular immune responses against the tumor antigen (1). Clinical studies with murine anti-id mAbs have demonstrated that the induction of a specific antibody response by anti-Id antibody vaccines is associated with prolonged survival of tumor patients in cases of malignant melanoma (1–3) and advanced colorectal cancer (1, 4, 5). Our studies confirmed these observations for the treatment of advanced ovarian carcinoma using the anti-Id vaccine mAb ACA125 (6–8).

The murine anti-Id ACA125 mAb mimics the epitope of CA125 antigen defined by the Id mAb (Ab1) OC125 (9). The CA125 antigen is a high molecular weight glycoprotein, recently identified as the mucin MUC-16 (10, 11), that is expressed in high amounts in about 80% of ovarian carcinomas. CA125 is physiologically expressed, in contrast, only in small amounts and is predominantly expressed in the uterus, endometrium, fallopian tube, ovaries, and serosa of the abdominal and thoracic cavity. Active immunization with tumor antigens, however, is limited by their typically low immunogenicity and the weakened immune capacity of tumor patients. On the other hand, anti-Id mAb ACA125 can effectively induce a specific anti-anti-Id antibody (Ab3) response against CA125 tumor antigen, as shown in animal models (9) and in clinical trials (6, 7, 8, 12). Because the titers of the Ab3 response correlate with prolonged survival of ovarian cancer patients after vaccination (8), there is a need to specifically increase the activity of ACA125-specific B cells without systemic B-cell stimulation.

IL-6 will be a suitable cytokine to increase B-cell maturation and function because IL-6 promotes B-cell and plasma cell differentiation, has profound effects on the synthesis of immunoglobulins, and is an essential growth factor for plasma cells (13, 14). Moreover, Burdin et al. (15) demonstrated that although maturing germinal center B cells do not produce IL-6, they express functional IL-6 receptors and proliferate in the presence of exogenous IL-6. Therefore, IL-6 present during the germinal center reaction is of high impact for the terminal differentiation of B cells.

To increase the Ab3 antibody response via exogenous IL-6, we evaluated two strategies in a mouse model: (a) coinjection of IL-6 together with the chimeric ACA125 antibody construct (chACA125) consisting of the anti-Id ACA125 scFv antibody (16, 17) joined to the human IgG1 CH2/CH3 domain; and (b) injection of the fusion protein chACA125-IL-6, which consists of the ACA125 scFv fused to human IL-6 via the IgG1 CH2/CH3 domain.

MATERIALS AND METHODS

Antigens and Antibodies. CA125 antigen (MUC-16) was purified from culture supernatants of CA125\(^+\) ovarian carcinoma OVCAR57 cells as described previously (18). Briefly, serum-free culture supernatant from OVCAR57 cells was precipitated with ammonium sulfate, dialyzed against PBS, and concentrated through a 50-kDa concentrator (Pall Gelman, Ann Arbor, MI). CA125 was determined by Elesys CA125 II Immunoassay (Roche Diagnostics, Mannheim, Germany). BSM (Sigma, Deisenhofen, Germany) served as a control antigen in antibody binding studies. Mouse mAb OC125 (CIS Bio International, Gif-Sur-Yvette, France) has specificity for the CA125 antigen (19); mAb ACA125 is a mouse anti-Id antibody to mAb OC125 (9). The unrelated mouse anti-Id mAb 14C5 Ab2 (20) was used as isotype-matched control.

Construction of the Fusion Proteins. The generation of recombinant ACA125 scFv has been described previously (16, 17). To generate the chACA125-IL-6 fusion protein, ACA125 scFv DNA was terminally linked by PCR with Xhol and BamHI restriction sites using 5\(^{'\prime}\)-GCCGC-CCAGTCTTAAATGCGCAGC-3\(^{'\prime}\) and 3\(^{'\prime}\)-ACCTGGATCCGGC-CGGTGATTTC-3\(^{'\prime}\)- and ligated into the eukaryotic expression vector pRSV-B72-3-scFv-γ (21) encoding a recombinant anti-TAG72 T-cell receptor. The DNA fragment for the anti-TAG72 B72-3-scFv binding domain was replaced by ACA125 scFv DNA. Human IL-6 cDNA was amplified by PCR from pT7/HIL-6 DNA (American Type Culture Collection) using 5\(^{'\prime}\)-GATCCAGATTCGGTATCACTAGGCCAGGAGAGATGCCAGGAGATTTC-3\(^{'\prime}\) and 3\(^{'\prime}\)-TCAACTCATTAGTTCATCATGCGCCACAGGACGCTCTTCC-3\(^{'\prime}\)- and ligated into the eukaryotic expression vector pRSV-B72-3-scFv-γ (21) encoding a recombinant anti-TAG72 T-cell receptor. The DNA fragment for the FceRIγ signaling domain was replaced by the IL-6 cDNA. Finally, the
human IgG1 CH2/CH3 cDNA sequence was amplified by PCR from the pRSV-BW431/26-CH2/3-γ plasmid (22) using 5′ primer (5′-CTGAG-GATCCC CGCCGAGCCCAATCTCCTGAAAAA AACT-3′) and 3′ primer (5′-CCCACCCA GATCTTTTATACCCGGAGACCA GGAGAG GCCTT-CTG-3′). This PCR product was digested with the restriction enzymes BamHI and BglII and inserted into the pRSV-ACA125-scFv-IL-6 plasmid between the ACA125 scFv and IL-6 sequences (Fig. 1). The chACA125 protein without IL-6 was generated as follows. The cDNA for the γ-signaling domain of the recombinant anti-CD30 receptor HRS3-scFv (23) was replaced by the DNA encoding the human IgG1 CH2/CH3 domains and the hinge region (Fc) as described above. For this purpose, the IgG constant domains were amplified and flanked with BamHI and Xhol restriction sites by PCR using the pRSV-BW431/26-CH2/3-γ plasmid DNA as template and the 5′ primer (5′-CTGAGGATCCC CGCCGAGCCCAATCTCCTGAAAAA AACT-3′) and 3′ primer (5′-GGCTTGAA CAGTATGATCTT ACCCGGAGACCA GGAGAG GCCT-3′). The chACA125 protein without IL-6 was generated by replacing the HRS3-scFv binding domain with ACA125 scFv DNA, which has been amplified by PCR and flanked with Xhol and BamHI restriction sites, respectively (Fig. 1).

Expression and Purification of Recombinant Antibody Constructs. Plasmid DNAs coding for chACA125-IL-6 and chACA125 were transfected into 293 cells using Effectene transfection reagent (Qiagen GmbH, Hilden, Germany). Briefly, 3 × 10^6 cells were transfected with 0.8 μg of plasmid DNA according to the manufacturer’s recommendations. Stably transfected cells were selected in the presence of G418 (2 mg/ml) and adapted to serum-free media containing 2% Ultraser-G (Life Technologies, Inc., Karlsruhe, Germany). The recombinant proteins were purified from culture supernatants by protein G affinity chromatography (Amersham Pharmacia Biotech, Freiburg, Germany).

SDS-PAGE and Western Blot Analysis of Recombinant Antibodies. Purified antibodies and fusion proteins were electrophoretically separated in an 8% polyacrylamide gel under reducing and nonreducing conditions. Proteins were either stained with Coomassie Blue or transferred onto a nitrocellulose membrane and probed with HRP-labeled goat antihuman IgG (1:1000; Fc-specific; Dianova, Hamburg, Germany) or, alternatively, with a biotinylated antihuman IL-6 antibody (1 μg/ml; Endogen, Woburn, MA) and HRP conjugated with streptavidin (1:1000; Endogen). Blots were visualized by chemiluminescence and autoradiography using the enhanced chemiluminescence Western blot detection system (Amersham Pharmacia Biotech).

Fig. 1. Expression vector for chACA125-IL-6 (A) and chACA125 (B) fusion proteins. LTR, Rous sarcoma virus long terminal repeat promoter; LK, α light chain leader; VH, heavy chain variable region; VL, light chain variable region; IgG CH2/3, human IgG1 hinge CH2 and CH3 constant regions. Restriction sites Xhol, BamHI, and XhoI are indicated.

Antigen Binding Assays. We tested binding of the chACA125-IL-6 and chACA125 fusion proteins to the Id mAb OC125 (Ab1) by ELISA. Briefly, microtiter plates were coated with mAb OC125 F(ab′)2, fragments (1 μg/ml), blocked, and incubated with the fusion protein for 1 h at room temperature. Bound fusion proteins were detected by incubation of a HRP-labeled goat antihuman IgG for 1 h at room temperature. Binding specificity of the chACA125-IL-6 and chACA125 fusion proteins was tested by competition assays using the mAb ACA125 or the CA125 antigen. Plates were coated with mAb OC125 F(ab′)_2 fragments (1 μg/ml) and incubated with fusion protein in the presence of increasing amounts of mAb ACA125 (0–10 μg/ml) or CA125 antigen (0–50 μl) units/ml). Additionally, the unrelated mouse anti-Id mAb 14CS F(ab′)2 fragments (0–10 μg/ml) and BSM (0–10 μg/ml) were used as controls. The percentage of binding inhibition was calculated as follows:

\[ % \text{inhibition} = \frac{100 \times \left(1 - \frac{\text{binding in presence of competitor}}{\text{binding in absence of competitor}}\right)}{1} \]

Assays for IL-6 Activity. IL-6 bioactivity of the chACA125-IL-6 fusion protein was determined using IL-6-dependent B9 cells. Briefly, B9 cells in RPMI 1640 and 10% FCS were incubated in 96-well microtiter plates (2 × 10^5 cells/ml; 100 μl/well) in the presence of increasing amounts of the chACA125-IL-6 fusion protein (0.15–78 pg) and, for control, the chACA125, the parental mAb ACA125, and rhIL-6 (specific activity, 100,000 units/μg; Roche Diagnostics). After 72 h at 37°C and in a 5% CO2 atmosphere, proliferation of B9 cells was quantified using the MTT cell proliferation kit (Roche Diagnostics) according to the manufacturer’s recommendations. Briefly, MTT reagent was added during the last 4 h of incubation. After solubilization, the absorbance was measured at 550 nm (reference, 660 nm). To verify that the bioactivity of the chACA125-IL-6 fusion protein is due to the IL-6 moiety, rhIL-6 (10 pg) and chACA125-IL-6 fusion protein (20 pg) were incubated together with increasing concentrations (0.001–1 μg/ml) of a neutralizing polyclonal rabbit antihuman IL-6 antibody (Endogen) or an unrelated rabbit IgG (DAKO, Hamburg, Germany) as control for 1 h at 37°C in a 96-well microtiter plate. After this preincubation step, B9 cells were added (2 × 10^5 cells/ml; 100 μl/well) and incubated for 72 h at 37°C and 5% CO2. The percentage of inhibition of proliferation was calculated as follows:

\[ % \text{inhibition} = \frac{100 \times \left(1 - \frac{\text{binding in presence of neutralizing antibody}}{\text{binding in absence of neutralizing antibody}}\right)}{1} \]

Immunization Studies. Female BALB/c mice (6 weeks old; 5 animals/group) each received i.p. injection with 50 μg of chACA125-IL-6 fusion protein suspended in complete Freund’s adjuvant. Mice were boosted three times at weeks 4, 7, and 10 using incomplete Freund’s adjuvant. For control, mice were immunized with 50 μg of the mAb ACA125, chACA125 fusion protein, and chACA125 protein cojected with 220,000 units of rhIL-6, respectively. The amount of cojected IL-6 corresponds to the IL-6 bioactivity of 50 μg of chACA125-IL-6 fusion protein as determined by proliferation assays (4,400 units of rhIL-6 correspond to 1 μg of chACA125-IL-6 fusion protein). Blood samples were collected 2 weeks after the second and third boosts.

Detection of CA125-specific Antibody (Ab3) Responses. Sera collected after the second and third boosts were screened by ELISA, Western blot analysis, and flow cytometry for a specific Ab3 antibody response directed against the CA125 antigen. Briefly, microtiter plates were coated with CA125 antigen (1000 units/ml) and with BSM (10 μg/ml) as control. Plates were blocked and incubated with sera of immunized mice or with mAb OC125 as positive control. Immune complexes were detected with HRP-labeled goat antihuman IgG (Fc specific; 1:2000). Antibody titers were defined as the highest dilution of sera with A405 nm > 0.1.

To evaluate specificity of the Ab3 response, CA125 antigen (500 units) was separated in a 8% SDS-PAGE under nonreducing conditions, blotted onto a nitrocellulose membrane, and probed with serum (diluted 1:1000) from mice immunized with chACA125-IL-6, chACA125 with or without soluble rhIL-6, and the parental mAb ACA125. mAb OC125 was added as positive control. Bound CA125-specific antibodies were detected with HRP-labeled goat antihuman IgG (Fc specific; 1:2000) and subsequently visualized by chemiluminescence techniques.

Binding of serum Ab3 to CA125 expressed on human ovarian cancer cells
was assessed by flow cytometry. Briefly, CA125<sup>+</sup> OAW-42 cells and CA125<sup>-</sup> SKOV-3 cells were incubated with mouse immune sera at a dilution of 1:500 for 1 h at 4°C. After washing, cells were stained with PE-conjugated rabbit antimouse IgG (DAKO) or 1 μg/ml rhIL-6 (Roche Diagnostics) and incubated with serial dilutions of immune sera, and bound antibodies were detected using a HRP-labeled goat antimouse IgG (1:2000). Antibody titers were defined as the highest dilution of sera with an A<sub>405nm</sub> > 0.1.

The capacity of antihuman IL-6 antibodies to neutralize the bioactivity of the fusion protein was determined by inhibition of B9 cell proliferation. To avoid nonspecific effects of serum components, the IgG fraction from mouse immune sera was purified by affinity chromatography using protein G. The chACA125-IL-6 fusion protein or rh-IL-6 was preincubated with increasing concentrations of purified mouse IgG (10–50 μg) from vaccinated mice for 1 h before adding to IL-6-dependent B9 cells. Proliferation of B9 cells was subsequently monitored as described above.

**Statistical Analysis.** The statistical significance of differences in antibody titers between experimental groups was determined by the unpaired Student’s t test using the SPSS analysis software 11.0.1 (SPSS Inc., Chicago, IL). Findings were regarded as significant with two-tailed Ps < 0.05.

**RESULTS**

**Generation of the chACA125 and chACA125-IL-6 Fusion Proteins.** DNA constructs coding for the chACA125-IL-6 and chACA125 fusion proteins were generated (see Fig. 1) and stably transfected into 293 cells as described in “Materials and Methods.” Recombinant proteins were secreted and purified from culture supernatants by affinity chromatography on immobilized protein G. We obtained 0.5–1 mg of chACA125-IL-6 and about 10 mg of chACA125 protein per 1 liter of culture supernatant (approximately 1–2 × 10<sup>6</sup> cells/ml) after 3 days. After electrophoretic separation under nonreducing conditions, the purified chACA125-IL-6 fusion protein migrated with an apparent mass of 170 kDa, and the chACA125 protein without the IL-6 domain migrated with an apparent mass of approximately 150 kDa, corresponding to the homodimeric forms of these proteins. Under reducing conditions, the proteins migrate electrophoretically as expected for the size of the monomeric proteins (Fig. 2A). Western blot analyses confirmed that both recombinant proteins contain the human IgG Fc domain, whereas the IL-6 moiety, as expected, is only present in the chACA125-IL-6 fusion protein (Fig. 2B).

**chACA125 and chACA125-IL-6 Bind to the Id mAb OC125.** The recombinant proteins were tested by ELISA for binding to the Id mAb OC125. As shown in Fig. 3A, both the chACA125-IL-6 and chACA125 proteins bind to mAb OC125 in a concentration-dependent manner. The binding capacity, however, is apparently lower than that of the parental mAb ACA125. Binding of the recombinant constructs to mAb OC125 was specifically blocked by both the parental mAb ACA125 (Fig. 3B) and the CA125 antigen (Fig. 3C), respectively. However, higher concentrations of the CA125 antigen are required for half-maximal inhibition of binding of the chACA125 protein (13 kilo units/ml CA125) compared with the chACA125-IL-6 fusion protein (5 kilo units/ml CA125; Fig. 3C). As expected, binding to mAb OC125 was not significantly inhibited by an unrelated anti-Id mAb 14C5Ab2 F(ab<sup>2</sup>)<sub>2</sub> fragment (0% inhibition) or by the control antigen BSM (inhibition for mAb ACA125, 61%; inhibition for chACA125, 5.4%; inhibition for chACA125-IL-6, 3.0%).

**chACA125-IL-6 Exhibits IL-6 Activity.** We tested the IL-6 bioactivity of the chACA125-IL-6 fusion protein using the IL-6-dependent cell line B9. As shown in Fig. 4A, the fusion protein sustains proliferation of B9 cells in a dose-dependent manner. Half-maximal proliferation of B9 cells was achieved with 12 pM chACA125-IL-6 fusion protein and 4 pM rhIL-6. Accordingly, the IL-6 activity of 1 μg of dimeric chACA125-IL-6 fusion protein corresponds to 4400 units of rhIL-6. As expected, mAb ACA125 and the chACA125 protein did not stimulate the proliferation of B9 cells (Fig. 4A). Proliferation of B9 cells is mediated by the IL-6 moiety of chACA125-IL-6 because the proliferation-sustaining activity of the fusion protein was inhibited by a neutralizing antihuman IL-6 antibody (Fig. 4B), but not by an unrelated control IgG (Fig. 4C).

**Antibody Response after Vaccination with the Recombinant Anti-Id Fusion Proteins.** We analyzed the Ab3 response in BALB/c mice immunized with (a) the recombinant chACA125-IL-6 fusion protein, (b) the chACA125 fusion protein with or without coinjection of rhIL-6, and (c) mAb ACA125 (5 animals/group for each). Recombinant fusion proteins as well as mAb ACA125 were used in equimolar amounts, whereas the amount of 220,000 units of rhIL-6 corresponds to the IL-6 bioactivity of 50 μg of chACA125-IL-6 fusion protein as determined by proliferation assays (4,400 units of rhIL-6 exhibit a proliferative response equivalent to that of 1 μg of chACA125-IL-6 fusion protein). Sera collected after the third boosts were analyzed for the presence of CA125-specific Ab3, antihuman IgG, and antihuman IL-6 antibodies, respectively. As summarized in Fig. 5A, immunization with the chACA125-IL-6 fusion protein resulted in high titers of Ab3 antibodies directed against the CA125 antigen, whereas preimmune sera did not harbor detectable titers of CA125-specific antibodies. Ab3 titers induced by chACA125-IL-6 fusion protein were significantly higher (P < 0.05) compared with immunization with equimolar amounts of mAb ACA125 or chACA125 protein with and without coadministered rhIL-6. In contrast, no significant binding of the mouse immune sera to the antigen-negative control mucin BSM was detected (Fig. 5B).

We additionally monitored the induction of antibodies against the xenogenic IgG Fc and IL-6 domain. Immunization with the recombinant chACA125-IL-6 or the chACA125 protein induced similar antihuman IgG titers in all animals (Fig. 5C). Interestingly, the presence of IL-6, either as part of the fusion protein or coadministered with the chACA125 protein, did not significantly affect the titer of antihuman IgG antibodies, whereas injection of the murine mAb ACA125, as
expected, did not induce antihuman IgG antibodies. In contrast, mice immunized with the chACA125-IL-6 fusion protein developed only low antihuman IL-6 titers, whereas rhIL-6 coinjected with chACA125 induced substantially higher antihuman IL-6 titers (P/H110210.0001; Fig. 5D). Obviously, the IL-6 domain is differentially presented when administered as rhIL-6 protein or as part of the chACA125 fusion protein. We further asked whether the higher anti-IL-6 titers of mice immunized with chACA125 plus rhIL-6 affect the IL-6 bioactivity differentially compared with chACA125-IL-6-immunized mice. We therefore tested whether the anti-IL-6 serum antibodies neutralize the bioactivity of IL-6. Antihuman IL-6 antibodies were affinity purified from sera of immunized mice as described in "Materials and Methods." When added to B9 cells stimulated with chACA125-IL-6 or rhIL-6, these anti-IL-6 antibodies did not affect B9 cell proliferation (Fig. 6). This indicates that the different Ab3 titers result from intrinsic properties of the chACA125-IL-6 fusion protein rather than from neutralizing anti-IL-6 antibodies.

Furthermore, we assayed the specificity of the chACA125-IL-6 protein-induced Ab3 response by Western blot analysis and flow cytometry using purified CA125 antigen and CA125+ tumor cells, respectively. Western blot analysis demonstrates that immune sera from mice vaccinated with the chACA125-IL-6 fusion protein detect the same 220-kDa CA125 tumor antigen as does the parental mAb OC125 (Fig. 7). Antibodies from immune sera, moreover, bind specifically to CA125+ OAW-42 human ovarian cancer cells, but not to CA125- SKOV-3 cells, as indicated by flow cytometry (Fig. 8C). Sera from nonvaccinated animals or unrelated mouse immune sera did not bind to CA125+ cells (Fig. 8, A and B). Remarkably, the same serum dilutions of animals that were immunized with the murine ACA125 mAb or the recombinant chACA125 with or without coinjected rhIL-6 did not react with CA125+ tumor cells (Fig. 8, D and E).

![Fig. 3. chACA125-IL-6 fusion protein binds specifically to the Id mAb OC125.](image)

A, microtiter plates were coated with mAb OC125 F(ab')2 fragments and incubated with serial dilutions of chACA125-IL-6, chACA125, or mAb ACA125, respectively. B, recombinant proteins were incubated in the presence of increasing amounts of mAb ACA125 in microtiter plates coated with mAb OC125 F(ab')2 fragments. C, plates were coated with mAb OC125 F(ab')2 fragments, and serial dilutions of the CA125 antigen were added together with constant amounts of chACA125-IL-6, chACA125, or the parental mAb ACA125, respectively. Bound proteins were detected with a HRP-conjugated antihuman IgG or antimouse IgG antibody. Percentage of inhibition was calculated as described in "Materials and Methods." Error bars indicate SD values of triplicates.

![Fig. 4. IL-6 bioactivity of chACA125-IL-6 fusion protein.](image)

A, IL-6-dependent B9 cells were incubated for 72 h in the presence of increasing amounts of the chACA125-IL-6 fusion protein, chACA125 protein, mAb ACA125, and rhIL-6, respectively. Proliferation of B9 cells was determined using the MTT assay as described in "Materials and Methods." B and C, neutralization of IL-6 bioactivity. rhIL-6 (10 pM) and chACA125-IL-6 fusion protein (20 pM) were preincubated with various concentrations of a rabbit antihuman IL-6 neutralizing antibody (B) or an unrelated rabbit IgG control antibody (C) before being added to IL-6-dependent B9 cells. After 72 h, proliferation of B9 cells was monitored using the MTT assay, and the percentage of neutralization was determined. SD values of triplicates are indicated by error bars.
or with electrophoretically separated CA125 antigen (Fig. 7). Accordingly, these sera obviously contain substantially lower Ab3 titers than the sera from animals immunized with chACA125-IL-6 protein.

DISCUSSION

Anti-Id antibodies are used as surrogate for tumor-associated antigens in vaccination trials that result in a specific antitumor response (1–9). Because a strong Ab3 antibody immune response is associated with prolonged survival of tumor patients, particularly in the case of ovarian cancer patients treated with the anti-Id vaccine ACA125 (8), there is a need to improve the potency of this vaccine. A number of strategies to enhance the immunogenicity of antigens by genetic engineering were reported, including the combination of antigen with proinflammatory chemokines (24) or toxins (25, 26). Because IL-6 increases B-cell maturation and antibody production, we have evaluated two strategies of immunization with the recombinant anti-Id vaccine ACA125 scFv in a mouse model: (a) coinjection of rhIL-6 together with the recombinant chACA125 antibody; and (b) injection of the recombinant fusion protein chACA125-IL-6. The fusion protein is designed to enhance specific activation of Ab3-expressing B cells and to avoid a general, nonspecific immune stimulation. Because dimerization of the antibody binding region is thought to result in enhanced binding avidity compared with a monomeric scFv (27, 28), we inserted the human IgG1 hinge-CH2CH3 domain into the fusion protein to induce an IgG-like dimerization of the molecule via disulfide bonds as shown by SDS-PAGE and Western blot analysis. Binding of the chACA125 and chACA125-IL-6 fusion proteins to the Id antibody OC125, however, was slightly impaired compared with

Fig. 5. Antibody titers after vaccination of BALB/c mice. BALB/c mice (5 animals/group) were immunized by i.p. injection with 50 μg of either the chACA125-IL-6 fusion protein, mAb ACA125, or chACA125 protein with or without coadministration of rhIL-6. After the third boost, the serum titers of anti-CA125 antibodies (Ab3), antihuman IgG1 antibodies, and of antihuman IL-6 antibodies were determined by ELISA using microtiter plates coated with CA125 antigen, BSM (control), rhIL-6, and human IgG antibodies. Bound antibodies were detected by a HRP-conjugated antimouse IgG. Antibody titers were defined as the highest serum dilution giving an A405 nm > 0.1 in the assay. Antibody titers in preimmune sera were found < 1:10. A, specific Ab3 titers after third boost. Differences between animals immunized with chACA125-IL-6 fusion protein and control groups are statistically significant (P < 0.05). B, anti-BSM antibody titer after the third boost (specificity control). C, anti-human IgG1 titers after third boost. Differences between groups receiving chACA125-IL-6 or chACA125 proteins with and without rhIL-6 are not significant. D, antihuman IL-6 antibody titers after the third boost. IL-6 as part of the chACA125-IL-6 fusion protein induced antihuman IL-6 antibody titers, which are significantly lower (P < 0.0001) compared with control mice immunized with chACA125 plus coadministered rhIL-6. Columns represent the mean titer of five identically treated animals, and the values are indicated above. n.d., not detectable. SD values are indicated by error bars.

Fig. 6. Antihuman IL-6 antibodies in mouse immune sera do not neutralize the IL-6 bioactivity. Fusion protein chACA125-IL-6 (20 psu) and rhIL-6 (10 psu) were preincubated with 10 μg/ml IgG purified from sera of preimmune mice or mice immunized with chACA125-IL-6, chACA125, or chACA125 plus rhIL, respectively, and added to B9 cells as indicator cells. Rabbit antihuman IL-6 neutralizing antibody (1 μg/ml) was used as control. After 72 h, proliferation of B9 cells was monitored by using the MTT assay, and the percentage of growth inhibition was determined as described in “Materials and Methods.” SD values of triplicates are indicated by error bars.
The IL-6 and addition, the molecular structure of the fusion protein itself may contribute to the observed differences in Ab3 induction. The IL-6 and fusion protein harboring the human IgG Fc and IL-6 domain in a single molecule is dominated by the antihuman IgG response. In contrast to the Ab3 response, we did not find significant differences in the titers of antihuman IgG antibodies after vaccination with the chimeric fusion proteins, whereas coinjection of rhIL-6 or immunization with the parental mAb ACA125. The specificity of the immune response induced by the chACA125-IL-6 fusion protein was shown by binding of Ab3 antibodies to the purified CA125 antigen as well as to CA125\(^+\) ovarian carcinoma cells (compare Figs. 5, 7, and 8). Strikingly, coinjection of rhIL-6 together with the chACA125 protein failed to increase the Ab3 titer significantly (Fig. 5A). We therefore conclude that the effect of the chACA125-IL-6 fusion protein is based on its particular design.

In contrast to the Ab3 response, we did not find significant differences in the titers of antihuman IgG antibodies after vaccination with the chimeric fusion proteins, whereas coinjection of the chACA125 fusion protein with rhIL-6 induces substantially higher antihuman IL-6 antibody titers than administration of the chACA125-IL-6 fusion protein. This suggests that induction of the Ab3 response and induction of the antihuman IgG Fc or antihuman IL-6 response may be based on different mechanisms. We speculate that the chACA125-IL-6 fusion protein may directly and with high efficiency stimulate the ACA125 idiotypic B cells by simultaneous binding of the scFv and IL-6 domains. In contrast, peptides derived from the xeno- genic, internal IgG Fc part of the fusion proteins are presented by dendritic cells with similar efficiency to both B- and T-cell receptor.

Similarly, coadministration of chACA125 and rhIL-6 likely result in antigen presentation to B and T cells independently of each other, followed by both a strong antihuman IgG and antihuman IL-6 response, whereas classical antigen presentation of the chACA125-IL-6 fusion protein harboring the human IgG Fc and IL-6 domain in a single molecule is dominated by the antihuman IgG response. In addition, the molecular structure of the fusion protein itself may contribute to the observed differences in Ab3 induction. The IL-6 and human Fc domains are directly joined together, probably preventing simultaneous binding of the human Fc domain and of the IL-6 domain to the same B cell, making direct activation of the antihuman IgG-specific B cell via fusion protein-bound IL-6 less effective. The same Fc domain of the fusion protein, on the other hand, may function as a flexible linker between the ACA125 scFv and IL-6 moiety of the protein, activating anti-Id-specific B cells more efficiently.

Recently, a clinical Phase I/II trial using ACA125 mAb as vaccine demonstrated that induction of Ab3 antibodies has a beneficial impact on the survival of patients with ovarian carcinoma recurrences (8). Notably, the survival benefit of Ab3-positive patients is not thought to result from a generally improved immunocompetence or a better prognosis because Ab3 responders are equally distributed among patients with 1 and \( \geq 3 \) previous chemotherapies. Other prognostic factors such as International Federation of Gynecologists and Obstetricians (FIGO) stage, response to first-line chemotherapy, or concomitant antitumor therapy did not significantly influence the observed immune response and clinical efficacy of the ACA125 mAb (8).

As a consequence, patients showing no specific antibody responses...
or only low specific antibody responses do not benefit from vaccination. Because our data suggest that the IL-6 domain of the anti-Id scFv fusion protein seems to act predominantly and specifically on the antigen-specific B cell rather than as a stimulator of an overall immune response, immunization with the chACA125-IL-6 fusion protein might be favorable over a mouse or human anti-Id vaccine in a clinical setting. Moreover, upon clinical application of the chACA125-IL-6 fusion protein, ovarian cancer patients are not expected to develop a nonspecific immune response against the human IgG1 CH2CH3 and IL-6 domains of the fusion protein as observed in the mouse model, resulting only in a highly specific and efficient Ab3 response. We therefore assume that antigen-IL-6 fusion proteins may be of general advantage in increasing the specific humoral immune response in anti-Id vaccination strategies.

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Interleukin-6 Fused to an Anti-idiotypic Antibody in a Vaccine Increases the Specific Humoral Immune Response against CA125\(^+\) (MUC-16) Ovarian Cancer

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