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Immunological properties of a single-chain fragment of the anti-idiotypic antibody ACA125

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Abstract Vaccination with anti-idiotypic antibodies has been described as a promising concept for treatment of several malignant diseases. The murine monoclonal anti-idiotypic antibody ACA125 imitates a specific epitope of the tumor-associated antigen CA125 expressed by 80% of ovarian carcinomas. In the first clinical trial it could be shown that mAb ACA125 is able to elicit anti-anti-idiotypic antibodies (Ab3) with anti-CA125 specificity in patients with advanced ovarian cancer. In order to improve the capabilities of anti-idiotype vaccines we generated a genetically engineered single-chain fragment (scFv) ACA125 composed of heavy- and light-chain variable regions connected by a flexible linker. The antigenicity of scFv ACA125 was demonstrated by immunizing rats i.p. with scFv or complete mAb in complete/incomplete Freund's adjuvants (CFA/IFA) or precipitated by aluminium hydroxide. Negative control groups included applications of irrelevant mouse IgG or adjuvants alone. Anti-anti-idiotypic antibodies (Ab3) directed against the mAb ACA125 as well as specific anti-CA125 antibodies (Ab1′) could be detected in all animals treated with scFv in CFA/IFA. Nevertheless, antibody titers were lower than when the complete mAb ACA125 was used. Suprisingly, an increase of specificity could not be observed in scFv-immunized animals, which had been expected because of the lack of heavy- and light-chain constant regions that could raise rather unspecific anti-isotypic and anti-allotypic rat anti-(mouse Ig) antibodies (RAMA). In contrast, the RAMA responses detected in these rats were even stronger than those following immunization with complete mAb ACA125. In conclusion, the anti-idiotypic scFv ACA125 alone cannot improve the immunogenic features of the corresponding mAb, but provides a useful tool for the further development of genetic vaccines.

Key words Anti-idiotype vaccination · scFv · Immune responses · Ovarian cancer

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Introduction

There is still no really effective treatment of ovarian carcinoma, despite the use of radical surgery and optimized polychemotherapy, and it therefore seems necessary to develop adoptive immunological approaches [19]. One promising principle of immunotherapy is the application of an anti-idiotypic antibody that imitates a critical epitope of the tumor-associated antigen CA125, expressed in about 80% of ovarian carcinomas [24]. The idiotypic network approach of N. Jerne indicates that such "internal-image" antibodies (Ab2b) are able to elicit a specific immune response against tumor tissue expressing the tumor-associated antigen [10], thus breaking the immunological tolerance against the tumor [3]. Immunization with anti-idiotypic antibodies should make activation of both the humoral and cellular branches of immunity possible. Anti-anti-idiotypic antibodies (Ab3) can be induced, which are directed against the anti-idiotype and simultaneously recognize the tumor antigen [9, 23]. Furthermore, these Ab3 could lead to complement-dependent cytotoxicity as well as antibody-dependent cytotoxicity. Presentation of anti-idiotypic antibodies through the MHC complex of antigen-presenting cells could raise a cellular immune response against CA125-positive tumors, which implies a rather unspecific natural killer (NK) activity or the induction of specific cytotoxic T cells [2].

The murine monoclonal antibody ACA125 (Ab2) is directed against the idiotypic mAb OC125 (Ab1) recognizing a specific epitope of the tumor antigen CA125. The complete mAb ACA125 has proved able to imitate

this CA125 epitope functionally in its antigenic determinants [17, 24].

In the first clinical trial, 45 patients with advanced ovarian carcinoma and recurrences were immunized with anti-idiotypic vaccine ACA125. Patients developing high amounts of specific anti-anti-idiotypic antibodies (Ab3) directed against CA125 have a significantly improved survival. This positive correlation between humoral immunity, indicated by the induction of Ab3, and the survival of cancer patients has also been shown in anti-idiotypic vaccination trials in cases of colorectal cancer [6, 7] and melanoma [14].

The limitation of adoptive immunotherapy by the use of anti-idiotypic antibodies lies in the difficulty of raising an adequate specific and homogeneous antitumoral immune response throughout a collective of cancer patients. In order to optimize anti-idiotypic vaccination we generated a genetically engineered single-chain fragment (scFv) composed of heavy- and light-chain variable regions of the anti-idiotypic mAb ACA125 connected by a flexible 15-amino-acid linker $(Gly_4Ser)_3$ [18].

The use of scFv fragments instead of the complete antibody may have two major advantages. On the one hand it could be expected that, because of the minimized structure of scFv, the induction of unspecific iso- and allotypic antibodies, predominantly raised against heavy- and light-chain constant regions, could be greatly reduced. In addition, these antibody fragments offer the opportunity to modify the entire variable sequence or to build new antibody constructs by fusion with immunoregulatory components in order to improve antigenicity as well as the specificity of anti-idiotypic vaccines.

ScFv fragments of monoclonal antibodies directed against tumor-associated antigens have already been functionally expressed and used in vivo as tumor-targeting agents for the directed delivery of toxins [13, 15] or cytotoxic T cells [8, 16] to the tumor microenviroment. In this context the capabilities of scFv have been widely discussed as far as binding affinities to the antigen, blood clearance or tumor penetration and retention are concerned. However, to our knowledge the capabilities of scFv to evoke specific antitumoral antibodies in vivo have been described only in cases of idiotypic scFv for non-Hodgkin's lymphoma [1] and anti-idiotypic scFv imitating the carcinoembryonic antigen (CEA) [22].

The aim of our investigations was to demonstrate the capability of scFv ACA125 to induce a specific anti-CA125 immune response in animals and compare this response to the effect of vaccination with complete anti-idiotypic Ab2. As the outcome of immunization may vary with the choice of carrier and adjuvants [4, 11], vaccination of the rats was carried out with two different adjuvants, complete/incomplete Freund's adjuvants (CFA/IFA) and aluminium hydroxide (alum).

Proof of the antigenicity of antibody fragments reduced to the variable regions would be important for the further design of anti-idiotypic constructs based on the ACA125 scFv.

Materials and methods

Production of the anti-idiotypic scFv ACA125

Cloning and expression of the anti-idiotypic scFv ACA125 in *Escherichia coli* by recombinant phage antibody technology [5, 12] was previously described [18]. Briefly, the variable heavy- and light-chain genes were amplified from hybridoma cells producing the mAb ACA125, connected by a 15-amino-acid linker $(Gly_4Ser)_3$ and cloned into the expression vector pCANTAB5E (Pharmacia). scFv expression was carried out in *E. coli* HB2151 cells in 1-l shake flasks. Functional scFv were harvested from *E. coli* periplasm and purified by affinity chromatography with NHS columns containing anti-Etag antibody (Pharmacia). ScFv was specifically bound to the column through the fused Etag peptide. The purity of the scFv preparation was determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 12.5% polyacrylamide gel under non-reducing conditions following Coomassie staining. Binding of scFv to the Ab1 OC125 was checked by enzyme-linked immunosorbent assay (ELISA). OC125 $F(ab)_2$ (1 μg/ml) was coupled to a microtiter plate and incubated with purified scFv in various dilutions. Bound scFv were detected with anti-Etag conjugated to peroxidase (POD) (Pharmacia; 1:4000).

Inhibition of binding of ACA125 scFv to OC125 $F(ab)_2$ was achieved by adding a mixture of fixed amounts of scFv (500 ng/ml) or mAb (100 ng/ml) and various dilutions of purified CA125 antigen (Biodesign; 1.25–125 kU/ml) to OC125-coated plates. Bound scFv were detected with anti-Etag–POD conjugate (1:4000), whereas mAb ACA125 was measured via rabbit anti-(mouse Ig)–POD conjugate (Dianova; Fc-specific, 1:2000). Inhibition of binding was calculated on the basis of the absorbance of sample without inhibitor.

Immunization of rats with ACA125 scFv

The immunological properties of the scFv in comparison to Ab2 ACA125 were examined in 6-week-old female Sprague-Dawley rats, which were immunized four times i.p. with 50 μg scFv, 100 μg mAb ACA125, 100 μg polyclonal mouse IgG (Dianova) or adjuvants alone (CFA/IFA, alum). Each group consisted of three identically treated rats. Injections were given in CFA/IFA, or the alum-precipitated preparation was used, every third week. Blood samples were collected after the second and third boost.

Detection of humoral immune responses

The induction of humoral immunity was monitored in sera of ACA125-immunized rats (scFv or mAb) after the second and third boosts. Animals treated with unspecific mouse IgG or adjuvants alone were taken as negative control groups.

The development of a rather unspecific rat anti-(mouse antibody) response (RAMA) including anti-iso, anti-allotypic as well as anti-anti-idiotypic antibodies (Ab3), was determined by a commercially available assay (Medac).

Specific anti-anti-idiotypic Ab3 in sera of immunized animals were detected by ELISA as described previously [17]. Briefly, ACA125 $F(ab)_2$ (1 μg/ml) was coated on a microtiter plate and incubated with sera or standard (Ab1 OC125) in various dilutions. Bound Ab3 (or Ab1) were detected with complete mAb ACA125 (1 μg/ml) and rabbit anti-(mouse Ig)–POD conjugate, Fc-specific (Dianova, 1:4000).

Binding of anti-anti-idiotypic Ab3 to CA125 antigen was measured by functional ELISA. CA125 (1000 U/ml) was immobilized on microtiter plates via lectin from *Tritium vulgaris* (10 μg/ml) and incubated with sera from immunized rats or standard Ab1 OC125. Immunocomplexes were detected as described above for measurement of Ab3 titers.

Detection of anti-anti-idiotypic Ab3 and anti-CA125 antibodies (called Ab1′) was carried out with rats' sera depleted of RAMA,

which were previously eliminated by selective binding to agarose-linked mouse IgG (Sigma). Ab3 and Ab1′ were quantified by OC125 Ab1 standards as OC125 arbitrary units (U) with 1 U/ml corresponding to 1 ng/ml bound OC125.

Inhibition of binding

To demonstrate the specifity of induced Ab3/Ab1′ responses the inhibition of binding to CA125 or ACA125 through the idiotypic mAb OC125 was studied.

Plates coated with CA125 or ACA125 $F(ab)_2$ were incubated with a fixed serum dilution containing Ab3 and various dilutions of the inhibitor OC125 $F(ab)_2$. Bound Ab3/Ab1′ were detected with goat anti-(rat Ig)–POD conjugate, Fc-specific (Dianova; 1.1000). The percentage inhibition was calculated on the basis of the absorption of sera without inhibitor.

Results

In vitro characteristics of anti-idiotypic ACA125 scFv

As described previously, the variable regions of the anti-idiotypic mAb ACA125 could be successfully cloned as a scFv (VH-linker-VL) in pCANTAB5E and functionally expressed in *E. coli* HB2151 [18]. Purification of ACA125 scFv from *E. coli* periplasm was achieved by affinity chromatography via the fused Etag peptide. As shown in Fig. 1 SDS-PAGE analysis of eluted fractions under non-reducing conditions revealed the presence of a single band of approximately 32 kDa representing the ACA125 scFv. The yield of purified scFv was estimated as that of a 200-µg/l *E. coli* shake culture with slight deviations depending on the batch of preparation.

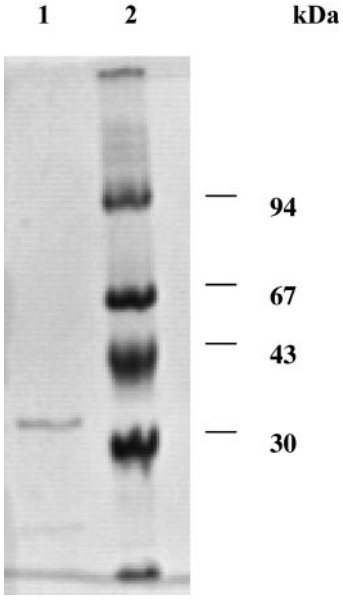

Fig. 1 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis analysis of ACA125 scFv under non-reducing conditions after Coomassie staining. *Lane 1* 1 µg ACA125 scFv purified from *Escherichia coli* periplasm by anti-Etag affinity chromatography, *lane 2* low-molecular-mass marker

The ability to bind the Ab1 OC125 could be clearly demonstrated for purified ACA125 scFv, although the reactivity of scFv was lower than of the corresponding mAb ACA125 (Fig. 2a). Furthermore, it could be shown that binding of ACA125 scFv to OC125 was almost completely inhibited by increasing concentrations of CA125 antigen (Fig. 2b). Thus, the anti-idiotypic scFv, like the complete mAb ACA125, binds specifically to the antigen-combining site of idiotype OC125, which otherwise recognizes the CA125 antigen. On the basis of these observations, the scFv ACA125 seems to retain the internal image characteristics of the complete mAb in vitro and might have the potency to mimic the CA125 antigen.

Induction of humoral immune responses by ACA125 scFv

In order to prove the capability of the anti-idiotypic scFv ACA125 to imitate the function of the tumor-associated antigen CA125, rats were immunized with scFv and mAb ACA125 in two different adjuvants (CFA/IFA

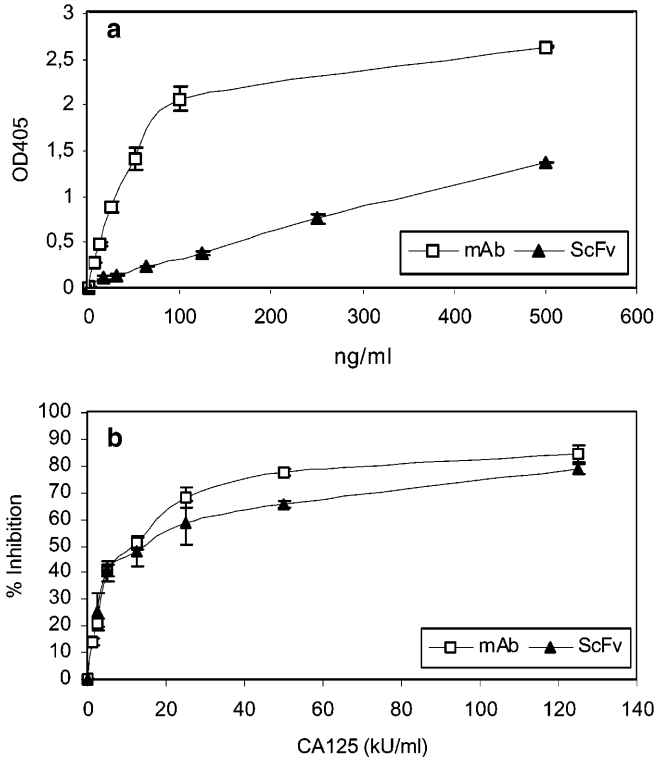

Fig. 2a, b Specific binding of the ACA125 scFv to Ab1 OC125. **a** Several dilutions of the purified scFv or mAb ACA125 were incubated on plates coated with Ab1 OC125 (1 µg/ml). Immunocomplexes were detected by peroxidase (POD)-labeled anti-Etag antibody (for scFv) or rabbit anti-(mouse antibody) (for mAb). **b** Inhibition of binding of scFv (in comparison to mAb ACA125) to Ab1 OC125 by CA125 antigen was performed by enzyme-linked immunosorbent assay (ELISA). Bound scFv or mAb ACA125 was determined as described above (**a**). Inhibition was calculated as a percentage of the value for sample without inhibitor CA125

and alum). Control groups received irrelevant mouse IgG or adjuvants alone to demonstrate the specificity of ACA125-induced immunity.

A rather unspecific RAMA response comprising anti-isotypic and anti-allotypic as well as anti-anti-idiotypic (Ab3) antibodies could be detected in all rats immunized with scFv ACA125 in CFA/IFA, whereas vaccination with the corresponding mAb in CFA/IFA resulted in an overall lower induction of RAMA (Fig. 3a). Alum-precipitated scFv and mAb ACA125 induced a weaker RAMA response than did the CFA/IFA preparation. The mouse IgG control group developed significantly higher RAMA titers than all the other groups (Fig. 3a).

Induction of anti-anti-idiotypic antibodies

Specific anti-anti-idiotypic Ab3 directed against the antigen-combining site of ACA125 could be detected in all scFv-immunized rats, but the titers were significantly lower than those of the complete mAb ACA125 (Fig. 3b). Vaccination with CFA/IFA or alum elicited equal amounts of Ab3, but a higher scatter of results was observed in rats immunized with complete mAb ACA125 in alum. As expected, the negative control groups (mouse IgG or adjuvants) showed no binding to ACA125.

The development of antibodies with specificity for the tumor-associated antigen CA125, the so-called Ab1′, could be demonstrated in rats after immunization with scFv ACA125 in CFA/IFA, whereas alum-precipitated scFv raised no anti-CA125 antibodies (Fig. 3c). Vaccination with complete mAb ACA125 resulted in higher Ab1′ responses within both groups (alum and CFA/IFA).

The ratio of Ab1′ (anti-CA125) induction to Ab3 (anti-ACA125) induction, after immunization with scFv or mAb, differs in different animals, so that high Ab3 titers cannot necessarily predict a strong anti-CA125 (Ab1′) response (Table 1). In addition, these data indicate that ACA125 scFv in CFA elicits overall a higher percentage of Ab1′ with anti-CA125 reactivity than does the corresponding mAb. In both groups, immunized with scFv and mAb, Ab1′ is induced later than Ab3 during the course of immunization, as shown by an increasing Ab1′/Ab3 ratio after the second and third boosts (Table 1).

Inhibition of binding

The specificity of anti-anti-idiotypic antibodies elicited by scFv and mAb ACA125 was shown by competition with Ab1 OC125.

Binding of antibodies from sera of scFv-immunized rats to the anti-idiotypic mAb ACA125 could be inhibited by Ab1 OC125 with a maximal competition of 23%, whereas the complete mAb ACA125 elicited Ab3 that could be almost completely blocked (80%–84%)

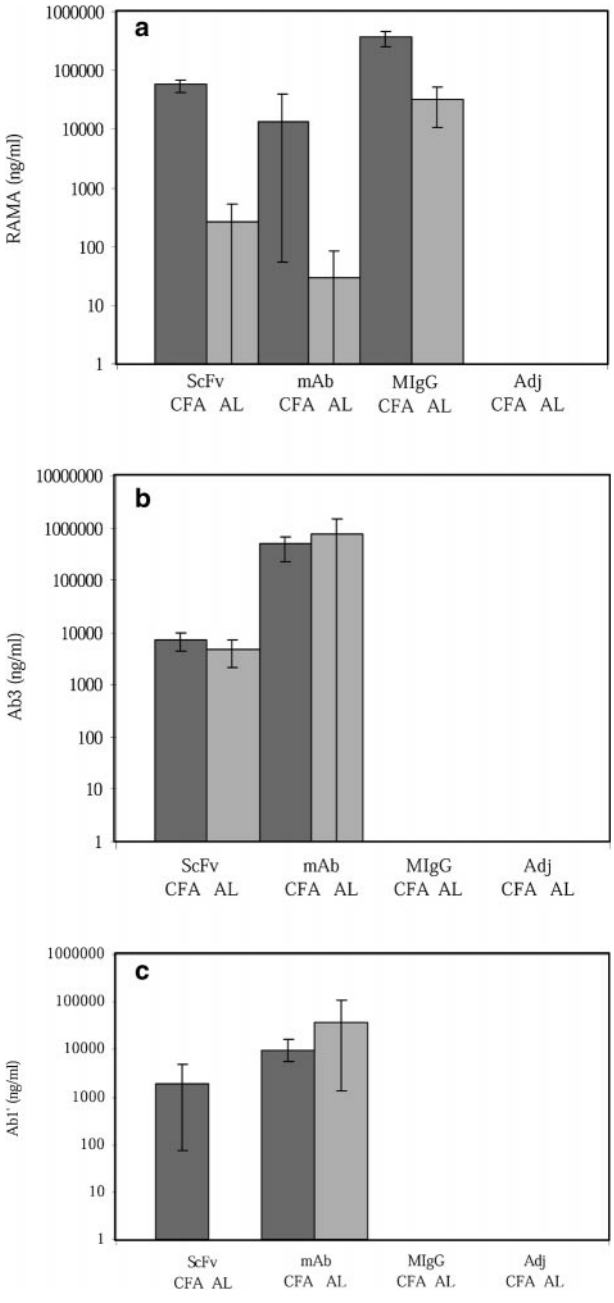

Fig. 3a–c Humoral immune responses in rats immunized with scFv or mAb ACA125 in complete Freund's adjuvant (*CFA*) or an alum-precipitated preparation (*AL*) after the third boost. Irrelevant mouse IgG and adjuvants alone were taken as negative controls. Columns represent mean values of sera from three identically immunized rats and ranges within one group are indicated. **a** Rat anti-(mouse antibody) response was determined by commercial ELISA (Medac). **b** Sera of immunized rats were incubated in plates coated with ACA125 $F(ab)_2$. Bound anti-anti-idiotypic Ab3 were detected by complete mAb ACA125 and rabbit anti-(mouse Ig) conjugated to POD. **c** CA125-specific Ab1′ titers were measured by incubating rats' sera on CA125-coated plates. Detection of immunocomplexes was achieved as described above

(Fig. 4). Negative control sera, derived from rats after injection of normal mouse IgG or adjuvants alone, showed no inhibition of binding to Ab2 by Ab1.

Table 1 Ratio of Ab1′ (anti-CA125) to Ab3 (anti-ACA125) in sera of rats immunized with scFv or mAb ACA125 in complete Freund's adjuvant (*CFA*) or alum-precipitated after second and third boost

Treatment	Rat	Ratio Ab1′/Ab3	
		After 2nd boost	After 3rd boost
ACA125 ScFv in CFA	1	1.395	0.615
	2	0.187	0.199
	3	0.008	0.009
ACA125 ScFv in alum	1	0	0
	2	0	0
	3	0	0
mAb ACA125 in CFA	1	0.005	0.008
	2	0.003	0.026
	3	0.078	0.028
mAb ACA125 in alum	1	0.023	0.143
	2	0.0004	0.001
	3	ND	ND

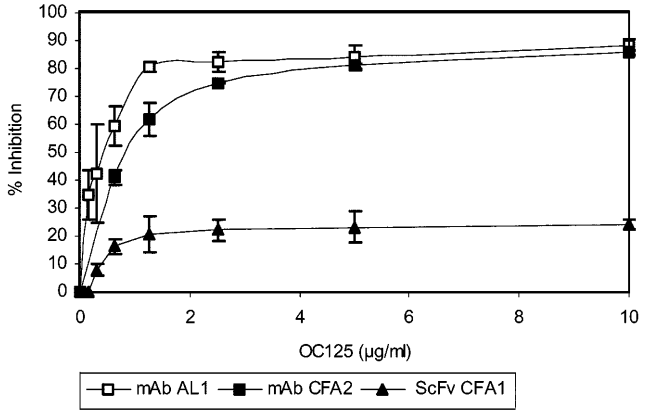

Fig. 4 Inhibition of Ab3 binding to Ab2 ACA125 by Ab1 OC125. A fixed dilution of sera from immunized rats (scFv or mAb ACA125, irrelevant mouse IgG in CFA/incomplete Freund's adjuvant and alum or adjuvants alone) after the third boost was incubated with different amounts of Ab1 OC125 on plates coated with Ab2 ACA125. Bound Ab3 were detected with POD-labeled goat anti-(rat Ig). Inhibition was calculated relative to sera without inhibitor

Therefore, it can be assumed that only a small percentage of Ab3 from the scFv-immunized rats can bind to the same Ab2 epitope as that recognized by Ab1. This implies that the majority of anti-CA125 antibodies detected after scFv vaccination bind to other structures of the CA125 antigen that are not imitated by the original mAb ACA125.

Discussion

The aim of this study was to define the minimum structural requirements for successful mimicry of the tumor-associated antigen CA125 by the anti-idiotypic antibody ACA125. Therefore, we constructed a scFv composed of the light- and heavy-chain variable regions of mAb ACA125 connected by a 15-amino-acid linker, which was expressed in *E. coli* [18] and subsequently characterized in vitro and in vivo.

Various scFv directed against tumor antigens have been described as retaining the ability to bind to the tumor antigen. Owing to their small size, scFv clear more rapidly from blood and penetrate faster and more deeply into tissues than whole antibody molecules [21].

Anti-idiotypic scFv for tumor therapy have to fulfill two different criteria. On the one hand, anti-idiotypic antibody fragments must still bind specifically to the idiotypic antibody, which implies a functional mimicry of the tumor antigen by the antigen-combining site. On the other hand, such vaccines have to be immunogenic to induce a specific antitumoral response. To our knowledge, only one study exists that demonstrates that an anti-idiotypic scFv is capable of successfully imitating a tumor antigen by inducing specific antitumoral antibodies in vivo [22]. To demonstrate these properties in the case of ACA125 scFv would be important for the development of improved recombinant anti-idiotype vaccines.

Our investigations show that the ACA125 scFv retained its ability to bind specifically to the idiotypic mAb OC125 in vitro, although the affinity was lower than that of the complete mAb ACA125. This phenomenon can be explained by the monovalent structure of scFv in contrast to the bivalent IgG. Competition assays clearly demonstrate that binding of ACA125 scFv to Ab1 OC125 was inhibited by increasing amounts of CA125 antigen in vitro. On the basis of this observation it could be concluded that the anti-idiotypic scFv recognizes the same structural epitope on OC125 as the nominal antigen CA125. Although these results indicate that the scFv ACA125 could be functionally expressed in *E. coli*, the overall yield obtained by the use of expression vector pCANTAB5E was unsatisfactorily low. The development of a more efficient scFv expression system, either by altering plasmid properties like promotor strength and leader sequences or by using intracellular expression and the isolation of scFv from *E. coli* inclusion bodies, should be taken into consideration. In addition, improved purification strategies based on affinity chromatography with immobilized idiotypic mAb OC125 would negate the need for the Etag peptide or alternative tagging-sequences, which perhaps could raise an inappropriate immune response in vivo.

Immunization of rats with ACA125 scFv instead of complete mAb ACA125 was conducted to prove the antigenic capabilities of an anti-idiotypic antibody fragment reduced to its variable regions. To amplify the immune responses we tested two different adjuvants, Freunds' adjuvant, as a known strong adjuvant, and the relatively weak alum, widely used for human applications. The humoral immune response in immunized rats was assessed by the detection of a rather unspecific rat anti-(mouse antibody) (RAMA) response, including anti-allotypic/anti-isotypic antibodies, and of specific

anti-anti-idiotypic antibodies. In general, it was found that immunization with alum as the adjuvant resulted in a higher scatter of induced immune responses between individual animals whereas CFA/IFA produced a more homogeneous response within the treated collective.

Because of the missing Fc part, it was expected that vaccination with scFv would result in lower unspecific RAMA responses than when the complete mAb ACA125 was used. Suprisingly, scFv in CFA/IFA, but not alum-precipitated scFv, induced an overall higher RAMA response than did the complete mAb. Thus, the majority of RAMA responses induced by scFv must be directed against framework regions of the variable domains. The induction of RAMA responses by administration of murine scFv could possibly be restricted to the immune repertoire of the specific rat strain used in our model system. We have learned from clinical trials with murine mAb ACA125 that the complete anti-idiotypic antibody develops high anti-(mouse antibody) (HAMA) titers in humans [24] but only low RAMA responses in Sprague-Dawley rats, as shown in the present study. On the basis of this observation it could be suggested that induction of unspecific anti-(mouse Ig) immune responses to scFv ACA125 might vary among different species in a similar manner.

Nevertheless, specific anti-anti-idiotypic Ab3 against the antibody-combining site of ACA125 were detected in all scFv-immunized rats regardless of the adjuvants, used, but the complete mAb ACA125 elicited higher Ab3 titers. Anti-anti-idiotypic antibodies with specificity for the CA125 antigen (Ab1′) could be observed after immunization with scFv in CFA/IFA but not with scFv in alum. Thus, detection of anti-CA125 antibodies in rats immunized with scFv ACA125 shows that the critical idiotope of mAb ACA125, mimicking the CA125 antigen, could be maintained in the scFv structure. In addition, recent data from vaccination with an scFv-ACA125-derived cytokine fusion protein (scFv ACA125 fused to interleukin-6) revealed that the induction of CA125 immunity by a recombinant scFv moiety is also possible in Balb/c mice (unpublished data) and therefore not limited by genetic restriction to individual species or strains.

As indicated by the ratio of Ab3 (anti-ACA125) to Ab1′ (anti-CA125), scFv-immunized rats exhibited a higher percentage of anti-CA125 antibodies than did rats treated with the complete mAb, but competition assays verified that only 20% of these antibodies recognized the CA125 epitope imitated by anti-idiotype ACA125. Thus, the majority of the Ab1′ induced by scFv seems to bind to other structures of the CA125 molecule that were not imitated by the original mAb ACA125.

Since idiotypic structures of Ab2 mimicking a tumor antigen are often conformation-dependent, this puts a special folding requirement in the expression system [20]. The folding of a linear scFv molecule in *E. coli* could lead to the exposure of amino acid residues other than those in the native antibody molecule, which would induce anti-anti-idiotypic Ab3 with a specificity different from that of CA125. As purification of ACA125 scFv from *E. coli* periplasm was achieved by selective binding to anti-Etag mAb directed against the Etag peptide fused to the scFv, it could be possible that the scFv preparation contained a mixture of protein folded to variable extents with only small amounts of real internal-image scFv capable of inducing specific anti-CA125 antibodies. According to this hypothesis, altering the purification strategy by using affinity columns with immobilized idiotypic mAb OC125 could lead to isolation of the functional internal-image scFv fraction.

In summary, these findings demonstrate that the anti-idiotypic scFv ACA125 reduced to the variable regions has the potential to mimic the CA125 tumor antigen in vivo, as proven by detection of anti-anti-idiotypic Ab3 and anti-CA125 antibodies in immunized animals. In comparison to the mAb ACA125 the corresponding scFv molecule induces an overall weaker and less specific immune response. Strategies to improve the specificity and antigenicity of scFv ACA125 could involve altering the purification scheme or genetic modifications towards humanization and fusion to immunogenic compounds (e.g. cytokines).

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