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In Vivo Studies with Covalent Conjugates of Cobra Venom Factor and Monoclonal Antibodies to Human Tumors

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A. Introduction

Cobra venom factor (CVF) is a nontoxic glycoprotein with $M_r \sim 140\,000$, obtained from cobra venom. CVF is a functional analog of C3b, the activated form of the third component of complement. Like C3b, CVF forms in serum with factor B of the alternative complement pathway an enzyme, the C3/C5 convertase [1]. The CVF-dependent enzyme is very stable and resistant to inactivation by control proteins. Therefore, once the enzyme is formed, it continuously activates C3 and C5.

We have previously shown that covalent conjugates of CVF with monoclonal antibodies to human tumor antigens are nontoxic by themselves, but elicit specific killing of antigen-positive tumor cells in the presence of serum complement in vitro [2]. We now wish to report our results of initial in vivo studies. We investigated the stability, the pharmacokinetics, and the tumor-suppressive activity of conjugates of CVF with the 9.2.27 murine monoclonal antibody to a human 250 000 daltons glycoprotein melanoma antigen [3].

B. Materials and Methods

CVF was purified from lyophilized *Naja naja siamensis* venom (Miami Serpentarium Laboratories) by sequential column chromatography [4]. Covalent conjugates of CVF with the 9.2.27 monoclonal antibody were prepared as described [2] with three different heterobifunctional cross-linking reagents: *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia), *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce), and iodoacetyl-*N*-hydroxysuccinimide ester (IAHS) (Fig. 1). IAHS was synthesized according to [5] with some modifications as described [6]. Prior to the coupling, CVF was radiolabeled with ^{125}I using immobilized chloramine-T (Pierce). For the pharmacokinetic studies, three female BALB/c mice each were injected i.v. with approximately 300 μg radiolabeled conjugates. At time intervals as indicated, the animals were bled and plasma samples were counted for radioactivity. Aliquots of the plasma samples were also subjected to 3%–9% gradient polyacrylamide gel electrophoresis in the presence of SDS with subsequent autoradiography.

C. Results

I. Pharmacokinetics and In Vivo Stability of Monoclonal Antibody–CVF Conjugates

Figure 2 shows a semilogarithmic plot of the elimination of the conjugates from mouse plasma. After an initial distribution

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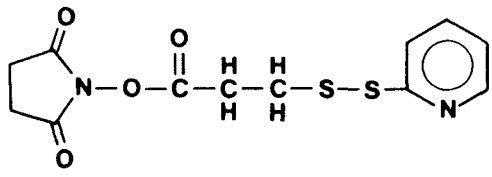
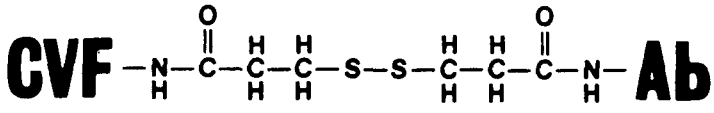
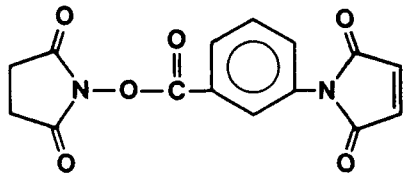
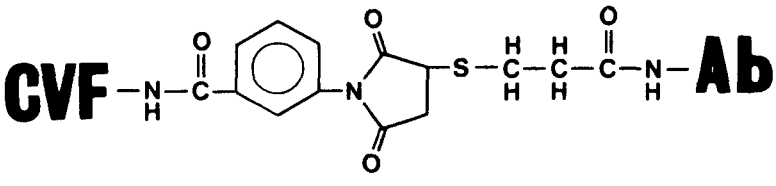
1. N-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP)



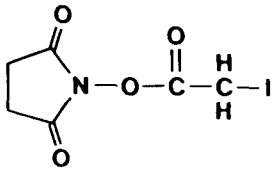


2. m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS)





3. Iodoacetyl-N-hydroxysuccinimide ester (IAHS)



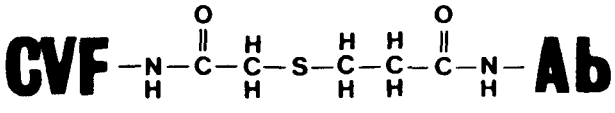


Fig. 1. Structures of the heterobifunctional cross-linking reagents and the resulting intermolecular cross-links. SPDP-linked conjugates contain a disulfide bond in the intermolecular cross-link while MBS-linked and IAHS-linked conjugates contain a thioether

phase of approximately 3 h, the SPDP-linked conjugates and IAHS-linked conjugates showed first-order elimination kinetics with plasma half-times of 23.5 and 25 h, respectively. In contrast, approximately 75% of the MBS-linked conjugates had been removed from the circulation within the first hour and continued to be eliminated with a half-time of 3.5 h. The fast removal of the MBS-linked conjugates was confirmed by the analysis of plasma samples by gradient gel electrophoresis and autoradiography. As shown in Fig. 3, MBS-linked conjugates were hardly detectable 10 h after injection while SPDP-linked and IAHS-linked conjugates were still present after 4 days. In addition, the higher oligomeric forms of the MBS-linked conjugates seemed to be preferentially removed.

Figure 3 also reveals that SPDP-linked conjugates underwent some degradation, as evidenced by an increasing amount of unconjugated CVF. However, this degradation was slow and the majority of the conjugates remained intact. A rather unexpected behavior was observed for the IAHS-linked conjugates. As evident from Fig. 3, the IAHS-linked conjugates exhibited in plasma a higher apparent molecular weight, suggesting a covalent interaction with plasma proteins.

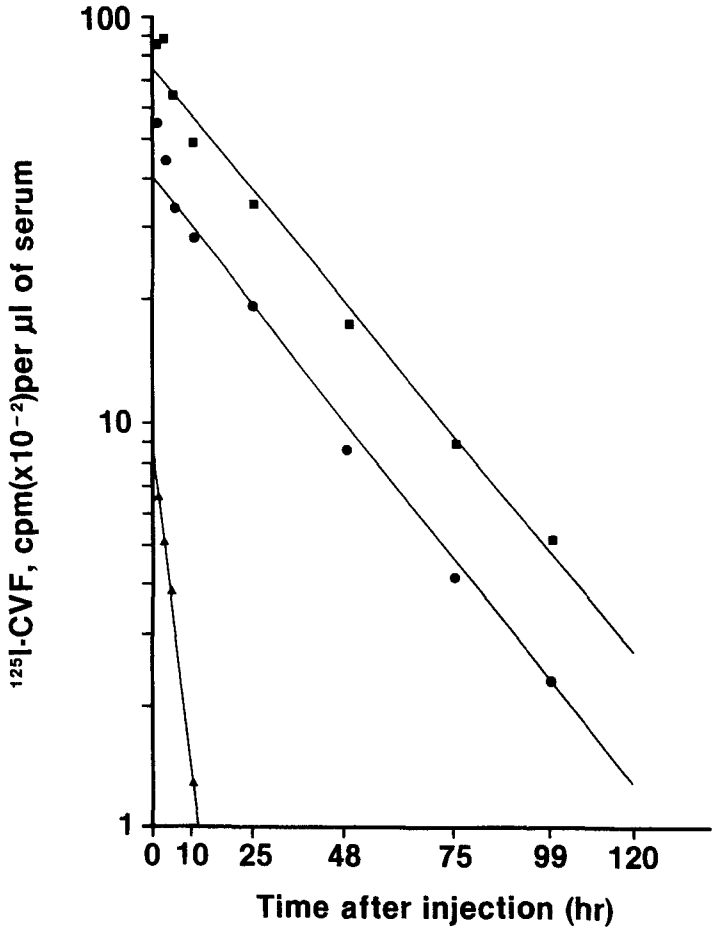


Fig. 2. Kinetics of elimination of 9.2.27 antibody-CVF conjugates from mouse plasma. Animals were bled at time intervals as indicated and plasma samples were counted for radioactivity (*squares* IAHS-linked conjugates; *circles* SPDP-linked conjugates; *triangles* MBS-linked conjugates)

II. Tumor-Suppressive Activity of Monoclonal Antibody–CVF Conjugates

Outbred nude mice with intraperitoneal transplants of human melanoma cells were used as a first model system to investigate the tumor-suppressive activity of monoclonal antibody-CVF conjugates. Mice were injected with 5×10^7 FEMX MET II cells. The animals received on the same day a single i.p. injection of 100 µg unconjugated 9.2.27 antibody or of SPDP-linked 9.2.27–CVF conjugates. Tumor growth, as evidenced by ascites formation, was delayed for approximately 2 weeks in animals treated with the 9.2.27–CVF conjugates compared with control animals treated with saline. The treatment with unconjugated 9.2.27 antibody was without effect.

D. Discussion

We synthesized covalent conjugates of the 9.2.27 monoclonal antibody with CVF, employing three different heterobifunctional cross-linking reagents. The stability and pharmacokinetics of these conjugates were investigated in mice. All three conjugates were, to a variable extent, stable in the circulation. Only the SPDP-linked conjugates, which contained a disulfide bond in the intermolecular cross-link, showed some degradation which is believed to occur by disulfide exchange or reductive cleavage (compare Fig. 1). However, the extent of this degradation does not preclude SPDP as a cross-linker for in vivo studies. MBS-linked conjugates were far more rapidly eliminated from the circulation than the two other conjugates. While the mechanism for this different kinetic behavior is not known, we believe that the hydrophobic nature of the MBS molecule may be responsible. IAHS-linked conjugates exhibited an interaction with plasma proteins, as demonstrated by their higher apparent molecular weight in plasma. This interaction with plasma proteins, which was also observed in vitro [7], must be covalent since it was resistant to boiling for 5 min in the presence of SDS. We are

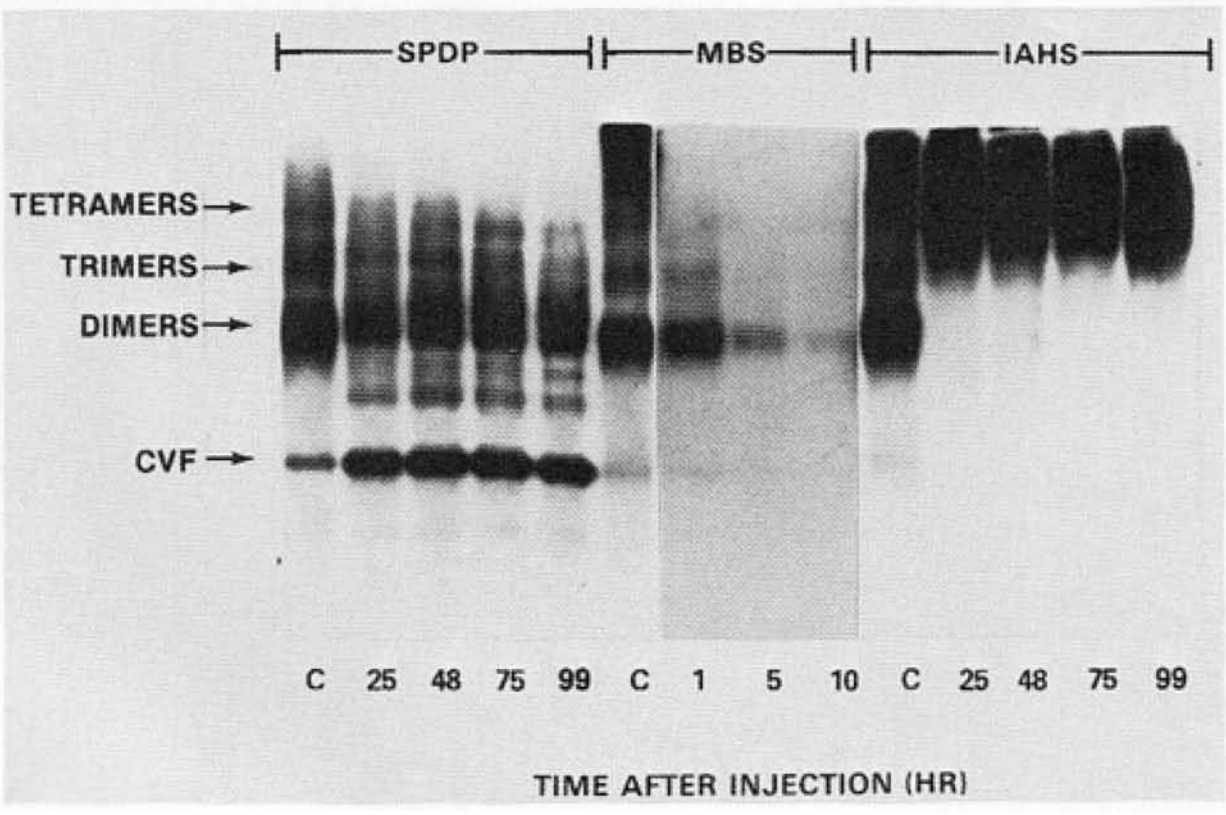


Fig. 3. Electrophoretic analysis of monoclonal antibody–CVF conjugates after injection into BALB/c mice. Shown is an autoradiogram of a 3%–9% gradient gel. *Control lanes* (marked *C*) show the injected material which contained dimeric, trimeric, and some higher oligomeric conjugates as well as small amounts of unconjugated CVF and antibody. Note that only CVF was radiolabeled

currently investigating whether unreacted IAHS groups remaining on the conjugates after the coupling reaction bind to plasma proteins containing free sulfhydryl groups or whether nascent C3b binds to the IAHS-linked conjugates through its reactive thioester.

Two important conclusions can be derived from our studies. First, stable conjugates of monoclonal antibodies with CVF exhibiting reasonable plasma half-times can be made. This finding now allows immunotherapeutic studies to be performed with such conjugates. Second, the nature of the heterobifunctional cross-linking reagent used for the synthesis of monoclonal antibody–CVF conjugates influences the pharmacokinetic behavior of the conjugates and, therefore, may have major impact on their distribution and immunotherapeutic effects.

We also performed an immunotherapeutic experiment in nude mice with intraperitoneally growing human melanomas. A single dose of 100 μg monoclonal antibody–CVF conjugates caused a significant delay of tumor growth after injection of a rather substantial inoculation of 5×10^7 melanoma cells. This promising result justifies further investigations of monoclonal antibody–CVF conjugates as potential agents for immunotherapy of cancer.

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