ESTIMATION OF NEOCARZINOSTATIN ACTIVITY IN CONJUGATES WITH MONOCLONAL ANTIBODIES

Sir:

Neocarzinostatin (NCS), a protein-like, antitumour antibiotic consisting of an apoprotein and an associated chromophore has been extensively characterized during recent years[1,2]. The mechanism of NCS action has been elucidated[3~5]. There is strong evidence that the non-protein chromophore intercalates, site-specifically between DNA base pairs and introduces single strand breaks and base release[6,7]. The chromophore becomes activated upon thiol addition and is then converted into an inactive species[8~10]. This reaction can be monitored by the shift in fluorescence excitation and emission properties of the drug[11,12]. NCS is very sensitive to light and heat and in its free from the chromophore becomes degraded very rapidly, especially at high pH[13].

Several groups have described the coupling of NCS to polyclonal and monoclonal antibodies to target the cytotoxic action to certain antigenic structures *in vitro* and *in vivo*[14~17]. Antibody-NCS conjugates have already been used in clinical trials[16].

The overall yield of active immunoconjugates is highly dependent on the amount of chromophore retained by the apoprotein as a result of the coupling procedure.

Little has been reported, however, on the composition of the conjugates in terms of coupled and still active NCS. The use of standard methods such as the agar diffusion[14,15] and the agar dilution[16,17] procedures are of doubtful value for analysis in which NCS linked to large proteins like antibodies is measured in the same way as free NCS, the reference standard[18].

In this communication we shall describe a comparison of 3 alternative and independent methods to determine the concentration of active NCS in conjugates with monoclonal antibodies. For each of these methods we have shown that: a) There is significant underestimate in the agar diffusion method, with NCS as a standard, b) that the procedures are precise and reliable in the estimation of NCS-molecules linked to antibodies.

Materials and Procedures

Clinical NCS generously provided by Kayaku Antibiotics (Tokyo, Japan) was used in this study.

Conjugates have been synthesized with the monoclonal antibodies 791T/36 and KG-6-56 *via* the heterobifunctional reagent *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)[19].

The products have been chemically and biologically characterized and stored in PBS-buffer, pH 6.5 at 4°C.

The agar plate diffusion test has been performed as described previously[15].

The diameters of growth inhibition were measured and plotted *versus* concentration of NCS as calculated from UV-spectroscopic measurements.

In an attempt to find out the best method for measuring the concentration of active NCS in conjugates, samples of free NCS and NCS-antibody-conjugates incorporating known drug concentrations according to the agar plate diffusion test, were subjected to three different analytical mehods: a) UV-absorbance at 280 and 340 nm, b) fluorescence intensity at 490 nm after excitation at 380 nm before and after chromophore extraction with 2-propanol, c) DNA-strand breaking activity using ^{3}H-labeled Col E1 plasmids. A biological assay has been designed to quantify the *in vitro* strand break activity of free as well as antibody linked NCS. For this purpose ^{3}H-labeled Col E1 plasmid DNA has been produced as described by MENKE *et al.*[20]. Samples of NCS-solutions were incubated in 60 mM NaCl, 6 mM Na-Citrate, 15 mM Tris-HCl and 0.15 mM DTT at pH 7.5 and 37°C. At various incubation times aliquots of 20 μl have been withdrawn from the reaction mixture and analyzed by agarose gel electrophoresis for loss supercoiled DNA[21]. The slope of the resulting curve can be taken as a measure of the amount of intact chromophore.

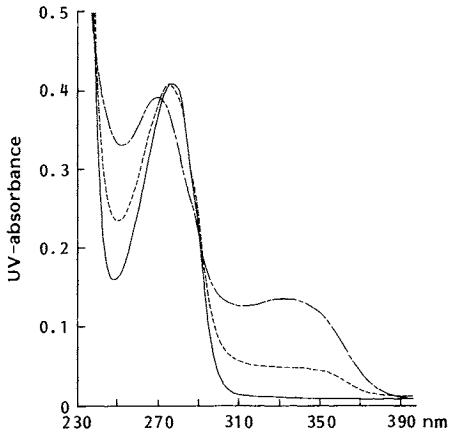
Results

a) The calculation of NCS/antibody ratio after conjugation is difficult for the 280 nm band due to additive UV-absorbance of both protein and chromophore. However, the concentration of the chromophore can be determined from its 340 nm absorbance (Fig. 1). Using the extinction coefficients for the NCS-chromophore ($e_{340}=8{,}000\,M^{-1}\,cm^{-1}$), apoprotein ($e_{280}=22{,}000\,M^{-1}\,cm^{-1}$) and antibody ($e_{280}=210{,}000\,M^{-1}\,cm^{-1}$)[11] the calculated concentrations in the example given are: NCS-chromophore $=4.4\times10^{-6}$ M and antibody $=1.2\times10^{-6}$ M. This result indicates an average substitution rate of 3.7 NCS per antibody.

This differs from the agar plate diffusion test result

Fig. 1. UV absorption spectra of free NCS, monoclonal antibody 791T/36 and NCS-antibody-conjugate.

—·—: 0.18 mg/ml in PBS, ——: 0.3 mg/ml in PBS, – – –: 0.3 mg/ml protein in PBS.



The spectra were measured on a Perkin-Elmer spectrophotometer model Lambda 5 (1 cm quartz cell).

which indicated only 0.5 NCS/antibody were found (data not shown).

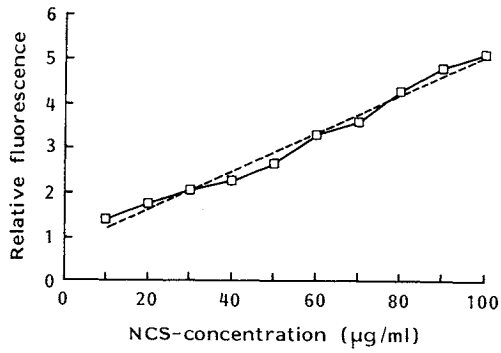
Consequently, the agar diffusion assay underestimated the concentration of the coupled drug by a factor of about 7.

b) Fluorescence intensity has been measured according to the method of POVIRK *et al.*[22]. The measurement of the relative fluorescence at 380/490 nm before and after 2-propanol extraction is directly related to the presence of the chromophore. Moreover we could show, that with the described method there is a linear correlation between the fluorescence intensity and the amount of biologically active NCS (Fig. 2). In the fluorescence assay two samples with NCS and NCS-conjugate both giving the same result in the agar plate diffusion test differ by the factor of about 9. Allowing for the overlapping of both emmission and excitation spectra for active and inactivated chromophore, the corrected value is about 7. This indicates that conjugates contain approximately 7 times more NCS than can be concluded from the agar diffusion method.

c) The comparison of the results in the DNA-strand breaking assay for NCS and NCS-conjugates, both producing the same inhibition diameter in the agar diffusion test, indicated that there is a underestimation by a factor of about 6 in the latter (Fig. 3).

We also tried to obtain exact data for NCS-concentration with the agar dilution method in 2-fold and 1.5-fold steps and conclude from the results that the test is unreliable (data not shown).

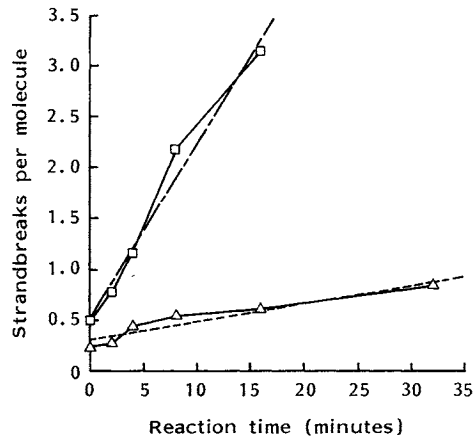
Fig. 2. Fluorescence calibration curve of NCS at various concentrations.



The fluorescence intensity of NCS-chromophore at Ex = 380 nm, Em = 490 nm before and after extraction with 2-propanol has been measured on the Perkin-Elmer fluorescence spectrometer type 650-10-S. The data points represent the means of triplicates. For details see text.

Fig. 3. DNA-strand breaking activities of NCS and antibody-NCS-conjugate.

□ NCS-conjugate, △ free NCS.



NCS and NCS-conjugate solution with the same inhibition zone diameter in the agar diffusion test indicating an apparent concentration of 10 μg/ml were diluted 1 : 10 and incubated with Col E1 plasmid DNA.

Strand breaks per molecule were plotted *versus* incubation time. The slopes for the curves differ by a factor of 6.2. See text for details.

Conclusion

1. The previously reported determination of NCS concentration in NCS-antibody-conjugates

with the agar plate diffusion assay underestimates the presence of active NCS by a factor of 6 to 7 times.

2. It is not the isolated chromophore that is the rate limiting molecule in the diffusion of the drug in agar inhibition tests, as has been assumed.

3. This evidence suggests that the reported increase in toxicity of NCS conjugated to monoclonal antibodies arises from the underestimate of its concentration in the assays previously used[15,16]. The substitution rate of the antibody has also been underestimated[17].

The toxicity of conjugated NCS is of the same order of magnitude as the free drug.

4. All three methods which we have described can be used for the measurement of NCS-concentrations in conjugates with monoclonal antibodies.

Acknowledgments

This work was partially supported by the Commission of the European Communities and by the Deutsche Forschungsgemeinschaft (DFG).

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(Received January 5, 1990)

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