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BIOLOGICAL ACTIVITY OF THE ANTITUMOR PROTEIN NEOCARZINOSTATIN COUPLED TO A MONOCLONAL ANTIBODY BY N-SUCCINIMIDYL 3-(2-PYRIDYLDITHIO)- PROPIONATE

Gundram Jung, Wolfgang Köhnlein and Gerd Lüders

Institut für Strahlenbiologie der Westfälischen Wilhelms Universität, 4400 Münster, Germany

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SUMMARY

The chromophore free apoprotein of neocarzinostatin was coupled to monoclonal IgG_1 antibody using N-Succinimidyl 3-(2-pyridyldithio)-propionate as heterobifunctional reagent. After coupling active chromophore was reassociated with the apoprotein. We present here experimental evidence that the hybrid protein retains biological activity as measured by the degradation of T2-DNA and bacteriostatic action.

INTRODUCTION

Although a large number of cytotoxic substances are known their lack of selectivity still represents a central problem in todays cancer chemotherapy. There have been many attempts in the past to increase the selectivity of anticancer drugs and the idea of directing toxins to cancer cells by using antibodies dates back to P. Ehrlich (1).

Recent developments in the technique of cellfusion and propagation of hybridoma secreting monoclonal antibodies of high selectivity for tumor cells made the construction of antibody-cytotoxin conjugates feasible (2,3,4). The toxic agents used so far, are small molecular weight standard cytostatic drugs (5,6) or high molecular weight toxins or their subunits (7,8,9). Cytotoxic agents especially of small molecular weight may loose their toxicity through the coupling process and only relatively few molecules can be bound to the antibody carriers without reducing their specificity. With the use of large molecular

Abbreviations: NCS, neocarzinostatin; apo-NCS, chromophore free neocarzinostatin; SPDP, N-succinimidyl 3-(2-pyridyldithio)-propionate; DTT, dithiothreitol; PBS, phosphate buffered saline; ABS, acetate buffered saline;

Dedicated to K.G. Zimmer on the occasion of his seventieth birthday

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weight toxins of high effectivity some of the difficulties have been surmounted, however, to release cytotoxic action in any case opening of a covalent bond is required.

Neocarzinostatin, a single chain acidic protein, has been shown recently to contain a non protein chromophore (10). This chromophore is not covalently bound to the protein and is the active part of the NCS (11,12). The chromophore alone degrades DNA after intercalation (13). The chromophore free apoprotein has no biological effect (14). The release of chromophore is the first step in neocarzinostatin action. This dissociation process which determines the biological availability of active chromophore can be influenced by various means (15). On that body of information we consider neocarzinostatin as suited for crosslinking to monoclonal antibody for the following reasons:

1) high effectivity of NCS chromophore on a molar basis as compared to other intercalating and DNA degrading drugs
2) chemical alterations of apoprotein do not influence the active principle (chromophore)
3) no opening of covalent bonds to release cytostatic action at the target
4) dissociation of chromophore can be controlled by adding or modifying apoprotein

With heterobifunctional reagent SPDP intermolecular crosslinks between proteins can be introduced employing the aminogroups of the involved proteins (16). In the case of NCS there are only two aminogroups available at alanin 1 and lysin 20. Since the first twenty aminoacid residues of NCS do not influence the ability of apoprotein to associate active chromophore (17), the biological activity of the drug should not be impaired by crosslinking NCS to monoclonal antibody using SPDP as coupling reagent.

We report here the successful crosslinking of NCS to an IgG_1 monoclonal antibody yielding hybrid proteins with biological activity. Experiments to test the specificity of such hybrid proteins for cells carrying the corresponding antigen are in progress.

MATERIAL AND METHODS

The antibody used is a IgG_1 myeloma protein obtained from the ascites fluid of pristan treated BALB/c mice and was gene-

rously given to us by R. Hentschel Dep. of Immunology, University, Münster. Neocarzinostatin (lot No. 770206) clinical form in 0.015 M sodium acetate buffer, pH 5, originated from Kayaku Antibiotics Research Co. and was kindly supplied to us by Dr. W.T. Bradner of Bristol Laboratories.

Heterobifunctional reagent N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and protein A-Sepharose Cl-4B were purchased from Pharmacia Fine Chemicals, Ampholines for the various pH-ranges were from LKB-Instrument. Thymine (methyl-^{3}H) (specific activity 20 Ci/mMol) from Amersham-Buchler was used to label T2-DNA.

The preparation of T2-DNA, sucrose gradient sedimentation and isoelectric focusing have been reported in detail elsewhere (18). The coupling procedures were performed according to the Pharmacia-Manual for SPDP. The different reaction steps are indicated in Fig. 1. After each step the reaction products were purified by dialysis and/or liquid chromatography (Sephadex G-25M). Rotary evaporation was used for concentrating. For isolation of IgG_1 antibody a protein A-Sepharose column was used as described by Ey et al. (19). Biological activity of NCS was measured by T2-DNA degradation (for details see legend of Fig. 3 and (18)) and inhibition of bacterial growth using the paperdisc method.

Apo-NCS was obtained by irradiating NCS with UV_{330} (total surface flux 9 KJ/m^2) and subsequent dialysis against 0.017 M acetate buffer pH 5 to remove dissociated chromophore. Material produced by this procedure yielded only one band in an isoelectric focusing gel (pI : 3.2) (20) and has the same absorption and fluorescence spectrum as chromatographically isolated apoprotein (unpublished results). Active chromophore was methanol extracted from lyophilized NCS as described by Povirk et al. (13).

RESULTS AND DISCUSSION

Ascites supernatant containing IgG_1 antibody was dialysed against 0.14 M PBS (pH 8) and loaded on a protein A-Sepharose column. Only material eluting at pH 6.0 was used for further treatment. As shown in Fig. 1 the first step of the coupling procedure is the reaction of SPDP with antibody which was performed in 0.1 M PBS (pH 7.5) at 28° C for 30 min with a 15 x molar excess of SPDP. This yields an average degree of substitution of 4-6 SPDP/antibody as measured by increase of $O.D._{343}$ due to the release of pyridine-2-thione after cleavage of disulfide bridges by dithiothreitol (DTT).

After the introduction of 2-pyridyl disulfide structures into the IgG_1 antibody unreacted SPDP and other low molecular weight reaction products were removed by dialysis and Sephadex G-25M columns. The SPDP-linked antibody was stored in 0.1 M PBS(pH 7.5). The protein concentration was about 1 mg/ml.

Since the coupling reactions have to be carried out under conditions favoring the release of chromophore (high pH and

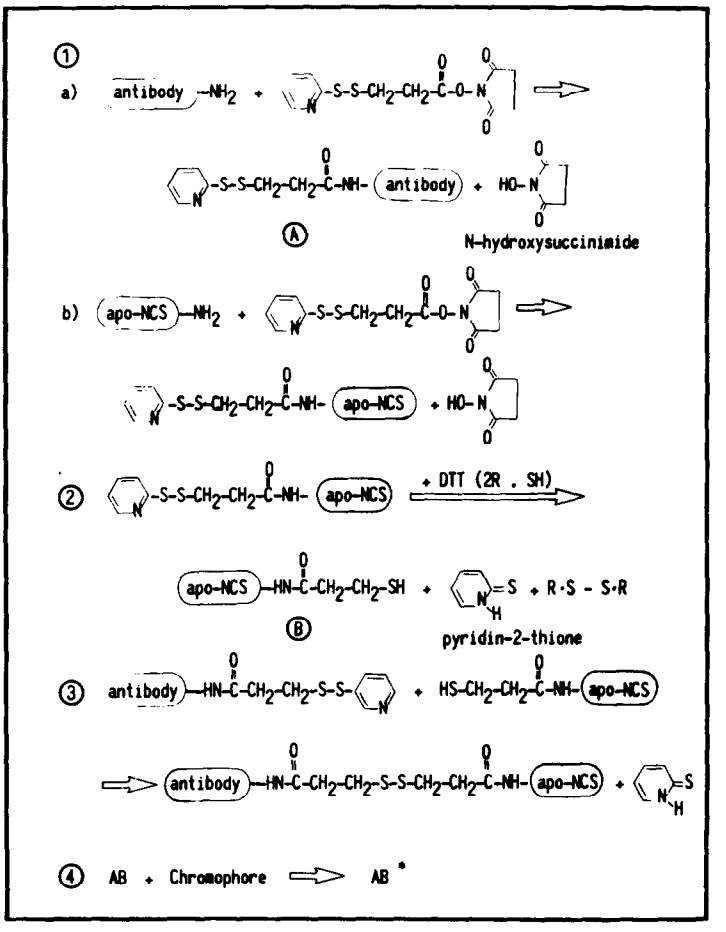

Fig. 1 Scheme for antibody-neocarzinostatin conjugation. 1. a) + b) Introduction of 2-pyridyl-disulfide structure into the proteins using SPDP. 2. Thiolation of the modified apo-NCS. 3. Conjugation of the proteins A and B. 4. Restoration of biological activity by reassociation of chromophore to antibody linked apo-NCS.

high thiol concentrations), the following reactions were performed with apo-NCS, making a final reassociation step with active chromophore necessary. Apo-NCS was linked to SPDP using the same conditions and the same excess of SPDP as described above (reaction 1b in Fig. 1) After coupling surplus SPDP and N-hydroxysuccinimide was removed by dialysis against 0.017 M ABS (pH 5.0) In our experiments the degree of substitution with SPDP was between 1 and 1.3 as measured by increase of $O.D._{343}$ after treatment with large excess of DTT. This thiolation is also the next step in the coupling procedure leading to free

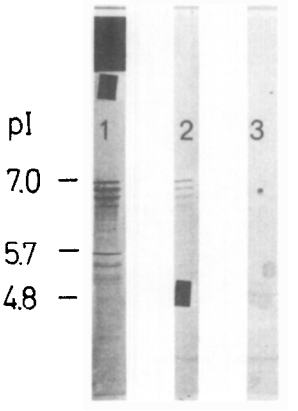

Fig. 2 Isoelectric focusing gels of IgG_1 monoclonal antibody: Ascites supernatant (1); after elution from a protein A-Sepharose column (2) and after coupling to SPDP (3). Gels contain 5% polyacrylamide and a crosslinkage of 3%. The final pH range was from 3.5 to 10. Focusing was for 16 h at 4° C; 3 mA initial current; 600 V final voltage.

SH-groups (reaction 2 in Fig. 1). After a reaction time of 30 min at 28° C surplus DTT was removed and the amount of free SH-groups available for the final coupling was measured with Ellman's reagent. In our experiments between 70% and 90% of the initial SH-groups are still available.

In the final conjugation of the modified proteins (reaction 3 in Fig. 1) for each SPDP residue bound to monoclonal antibody a two fold excess of reduced apoprotein bound SPDP was added. The reaction was carried out at 28° C for 24h (pH 7). The release of pyridine-2-thione, an indication of successful coupling, was monitored spectrophotometrically. Between 4 and 5 apo-NCS-molecules were linked to each monoclonal antibody. In a last step the conjugate was purified on a protein A-Sepharose Cl-4B column.

For restoring the biological activity of NCS a reassociation of active chromophore with antibody bound apoprotein was necessary. Isolated chromophore was added at high excess (x 10-20) at pH 5 for 30 min at 10° C (reaction 4 in Fig. 1). The reaction mixture was filtered on a Sephadex G 25 column and extensively dialysed against 0.1 M PBS,(pH 7.5) to ensure complete removal and decay of free chromophore. Isoelectric focusing patterns of monoclonal IgG_1 antibody after the various reaction steps are given in Fig. 2. These results can also be taken as an indication

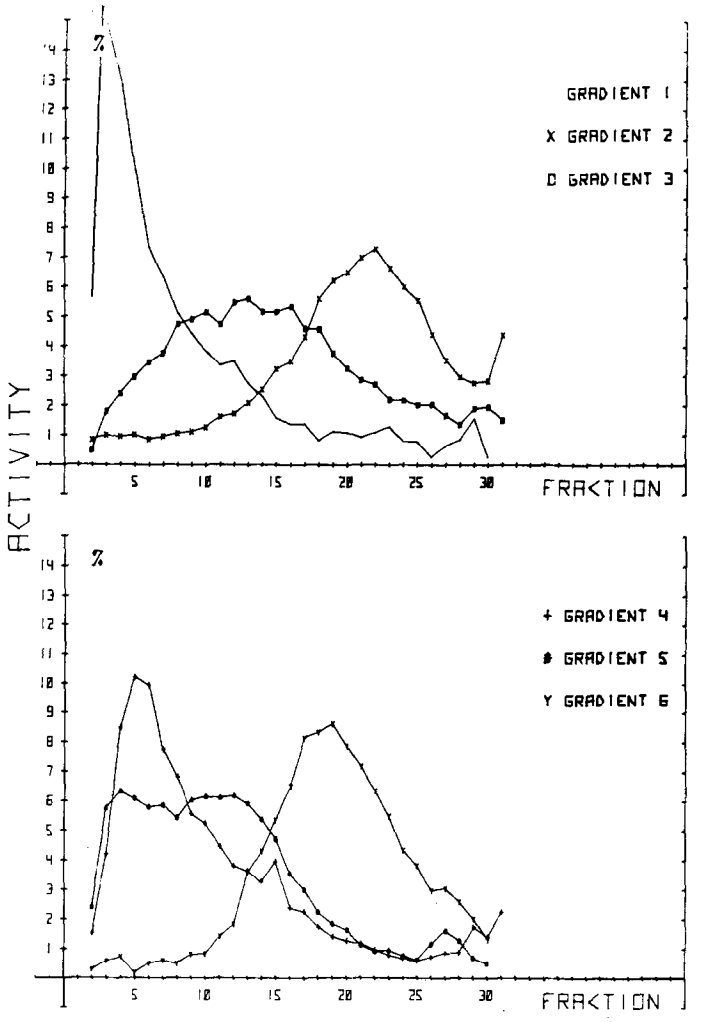

Fig. 3 Sedimentation pattern of T2-DNA in alkali sucrose gradients (110 min; 40.000 rev/min; 16° C; SW 50.1) after various treatments: (1) untreated; (2) treated with reassociated antibody bound NCS; (3) treated with active NCS 2 µg/ml final concentration under identical experimental conditions; (4) treated wich antibody bound apo-NCS; (5) treated with inactive apo-NCS; (6) treated as (2) in the presence of surplus apo-NCS.

of a successful modification of the antibody. The pI-points change from 7.0 for ascites supernatant and protein A-purified antibody to 4.8 for SPDP-antibody.

In Fig. 3 the sedimentation profiles of T2-DNA treated with NCS, inactive apo-NCS, reassociated antibody bound NCS and antibody bound apo-NCS are compared with untreated T2-DNA. One DNA sample was treated with antibody bound NCS in the pre-

sence of surplus apo-NCS. These results clearly indicate that antibody bound apo-NCS has no DNA degrading activity whereas the reassociated antibody bound NCS degrades DNA.

Upon addition of apo-NCS to antibody bound NCS the well known antagonistic effect is observed by the less pronounced degradation of DNA (20). A quantitative evaluation of the sedimentation profile according to the method of Litwin (21) yields a DNA degradation in sample (3) equivalent to a treatment with an NCS concentration of about 20 µg/ml. This is in fair agreement with the amount of NCS bound to antibody as estimated from the reaction conditions. These results are corroborated by the antimicrobial activity of NCS and antibody bound NCS, which was tested on a UV sensitive B. subtilis strain, UV_{ss} 19-8 (22).

We have shown, that neocarzinostatin can be linked to monoclonal antibody without loosing its biological activity. Since the hybrid protein still binds to protein A a drastic change of antibody integrity is unlikely. The antigen binding ability of the hybrid protein, however, remains to be shown. Coupling of NCS to monoclonal antibodies directed against defined cell surface antigens are in progress with the aim to yield specific toxicity to cells carrying the corresponding antigen.

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