Jpn. J. Cancer Res. 84, 1190–1194, November 1993

Production, Binding and Cytotoxicity of Human/Mouse Chimeric Monoclonal Antibody-Neocarzinostatin Conjugate

Toshiharu Yamaguchi,[1] Hiroshi Tsurumi, Kazuya Kitamura, Eigo Otsuji, Takuya Miyagaki, Tatsuya Kotani and Toshio Takahashi

First Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kawaramachi hirokojikajii-cho, Kamigyo-ku, Kyoto 602

A human/mouse chimeric Fab monoclonal antibody A7 (chFabA7) was covalently coupled to neocarzinostatin (NCS) by the SPDP method at various chFabA7:NCS substitution ratios. The antigen-binding activity of the conjugate, examined by ELISA using fixed antigen-positive colon cancer cells, was identical to that of the parent chFabA7 when one mole of NCS was conjugated, but was reduced with 2 or 3 moles of conjugated NCS. By means of a colony-forming assay, the cytocidal effect of the conjugate on antigen-positive cancer cells was found to be stronger than that of free NCS, whereas in antigen-negative cancer cells it was similar to that of free NCS. This effect was attenuated by adding an excess amount of monoclonal antibody A7. These findings indicate that the conjugate has an antigen-specific cytocidal action, and thus chFabA7-NCS is a promising tool for targeting cancer chemotherapy.

Key words: Mab A7 — Chimeric antibody — Drug conjugate

The use of antibodies as a carrier of pharmacological agents has become more practical since the advent of monoclonal antibody (Mab) technology, and several studies have demonstrated the potential use of immunoconjugates in clinical application.[1-5] We have reported immunotargeting chemotherapy using a murine Mab A7-drug conjugate for colorectal cancer patients.[6] One problem that has been encountered with the use of murine Mabs is the development of immune response to the administered antibody. This immune response has led to changes in antibody pharmacokinetics and more serious problems such as anaphylaxis.[7] There is also a problem with the limitation in tumor penetration of whole Mab, since a macromolecule the size of an immunoglobulin encounters physiological barriers, such as poor extravasation.

To overcome the drawbacks associated with murine whole antibody use, a new type of antibody is required. The new antibody should have properties such as reduced immunogenicity, small molecular size and sufficient antigen-binding activity. Recent progress in genetic engineering techniques has made it possible to construct an engineered antibody which can meet these requirements. This report describes the construction and *in vitro* properties of a chimeric Fab Mab A7 (chFabA7)-anticancer drug conjugate. To our knowledge, this is the first report describing the development and characterization of an engineered Mab-anticancer drug conjugate.

[1] To whom request for reprints should be addressed.

MATERIALS AND METHODS

Preparation of antibody, chemicals and cell lines Mab A7 was developed as described previously.[8] Mab A7 is known to react selectively with human adenocarcinoma[9] and to recognize a 45000-dalton glycoprotein on the cell surface.[10] chFabA7 was generously provided by Green Cross Co. Ltd. (Osaka). Details of the chFabA7 preparation will be described in a future report. The binding activity of chFabA7 to cells of a human colorectal cancer cell line was identical to that of the Mab A7 Fab fragment produced by papain digestion.[9] Neocarzinostatin (NCS), an anticancer polypeptide, was obtained from Pola Chemical (Yokohama). The activity of NCS conjugates is expressed in units equivalent to 1 μg of NCS. Heterobifunctional reagent, N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), .and dithiothreitol (DTT) was purchased from Wako Pure Chemicals Industries Co. Ltd. (Osaka).

A human colon cancer cell line, HLC-1, and human squamous cell cancer cell line, HeLa S3, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). HLC-1 and HeLa S3 cells were used as antigen-positive and antigen-negative cancer cells, respectively.

Production of chFabA7-NCS The conjugation procedures were generally carried out according to the Pharmacia Manual for SPDP. But modifications were necessary in order to retain NCS activity throughout the conjugation process and to minimize the formation of polymerized antibody-NCS.

Step 1: chFabA7 (10 mg/ml in phospate buffer, pH 7.20) was mixed with SPDP in ethanol, in a 1:1 molar ratio. The reaction mixture was stirred at 25°C for 30 min, then desalted on Sephadex G25 (Pharmacia) equilibrated with 0.1 *M* phosphate-buffered saline (PBS), pH 7.2. The resultant chFabA7 conjugated with 3-(2-pyridyldithio)propionylate (PDP) was collected as the excluded fraction from the column.

Step 2: NCS (20 mg/ml in Tris-HCl, pH 7.0) was mixed with an excess of SPDP in a 1:1.5 molar ratio. The mixture was stirred at 25°C for 30 min, then loaded onto Sephadex G 25 equilibrated with 0.02 *M* Tris-HCl, pH 6.5. The excluded fraction was collected, and NCS-1PDP was separated on a Q-Sepharose (Pharmacia) column with 0.02 *M* Tris-HCl, pH 6.5, containing a gradient from 50 *M* to 300 m*M* NaCl. The NCS-1PDP fraction was thiolated by stirring with excess DTT at 25°C for 30 min. The reaction mixture was purified on a Sephadex G25 column and the NCS-SH fraction was collected.

Step 3: chFabA7-PDP was combined with an excess of NCS-SH in a 1:1.5 molar ratio and the mixture was dialyzed against 0.05 *M* Tris-HCl, pH 7.0. The mixture was stirred at 25°C overnight, and loaded onto a Q-Sepharose column; elution was conducted with 0.02 *M* Tris-HCl buffer, pH 6.5, containing a gradient from 50 to 250 m*M* NaCl.

Binding activity of the conjugates The antigen-binding activities of the conjugates were measured by the competitive direct immunoperoxidase method using an HLC-1 cell monolayer. Aliquots of HLC-1 cells in growth medium (2×10^4 cells/well) were plated in sterile tissue culture microtiter plates and cultured for 48 h at 37°C. After removal of the supernatant, each well was filled with fresh growth medium and the plate was incubated for 2 h to block nonspecific protein-binding sites on the surface of the wells. The medium was removed by decantation, and 50 μl of the conjugate solution serially diluted in growth medium was added to each well. The wells were incubated with 50 μl of peroxidase-labeled Mab A7 Fab fragment (PO-A7 Fab) and 50 min later were washed four times with PBS containing 1% FCS. One hundred and fifty μl of substrate solution (0.05% *o*-phenylenediamine, 0.02% H_2O_2, citrate phosphate buffer, pH 5.0) was added to the wells and incubated for 30 min at room temperature in the dark. The reaction was terminated by adding 5 *N* H_2SO_4, and the optical density of each well was read at 492 nm using fresh substrate solution as a blank.

Cytotoxicity of the conjugates Cytotoxicity of the conjugates was determined using a colony formation assay. Fifty cells of HLC-1 or HeLa S3 in the medium supplemented with 8% FCS were plated in a 96-well culture dish and cultured for 24 h at 37°C in a humidified atmosphere of 5% CO_2. After removal of the supernatant, aliquots of 50 μl of medium containing serially diluted conjugate or NCS were added to the wells, giving final NCS concentrations ranging from 10 to $10\times(1/2^{10})$ U NCS/ml. After 30 min of incubation, the wells were rinsed twice and cultured at 37°C. Six days later, the dish was rinsed with 0.9% saline and the cell colonies were then fixed and stained with 1% crystal violet in methanol. The colonies were counted using a stereoscopic microscope and the results were expressed as the percentage of colonies formed in the wells treated with conjugate or free NCS, in comparison with the wells treated with the medium alone.

To confirm the antigen specificity of the cytotoxicity of the conjugate, a separate experiment was undertaken. In this study, antigen-positive HLC-1 cells on a 96-well plate were incubated with serially diluted chFabA7-1NCS or NCS in the presence of an excess amount of whole Mab A7 and non-specific IgG. After 30-min incubation, the wells were rinsed twice with 0.9% saline and cultured at 37°C. Six days later, the plate was rinsed and the cell colonies were fixed and stained with 1% crystal violet in methanol. The colonies were counted using a stereoscopic microscope and the results were expressed as the percentage of colonies formed in the wells treated with conjugate or free NCS.

RESULTS

Conjugate production chFabA7 was successfully conjugated to NCS through a disulfide bond by using SPDP, with various substitution ratios. The gel chromatography of the conjugate mixtures, performed using Q-Sepharose and Sephacryl S-200, yielded three distinct types of conjugates: 1-mol NCS form (chFabA7-1NCS), 2-mol NCS form (chFabA7-2NCS) and 3-mol NCS form (chFabA7-3NCS) (Fig. 1). SDS-PAGE (gradient gel from 4 to 20%) confirmed the purity of the conjugates (Fig. 2).

Binding activity of the conjugates The antigen-binding activity of the conjugates was investigated by a competition assay between PO-A7 Fab and each conjugate. Results were expressed as percentage binding of the serially diluted conjugates in comparison with the control. The density of bound PO-A7 Fab, when coincubated in buffer solution in place of the conjugates, was defined as 100% binding. The binding activity of chFabA7-1NCS was almost identical to that of parent chFabA7, but was markedly reduced in the chFabA7-2NCS and -3NCS conjugates (Fig. 3). The 50% binding concentrations of the conjugate were 2.5, 3.0, 5.5 and 10.0 for chFabA7, chFabA7-1NCS, chFabA7-2NCS and chFabA7-3NCS, respectively.

Cytotoxicity of the conjugates Conjugate cytotoxicity was investigated by colony formation assay. Data were

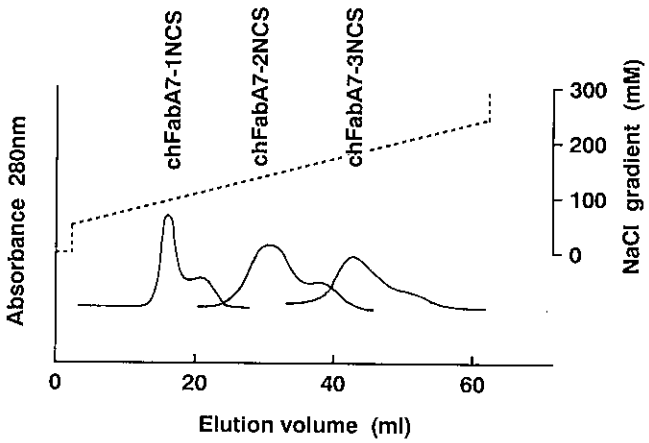

Fig. 1. Elution profile of a reaction mixture of chFabA7-PDP and NCS-SH using Q-Sepharose column chromatography. The first, second and third peaks are due to chFabA7-1NCS, chFabA7-2NCS and chFabA7-3NCS, respectively.

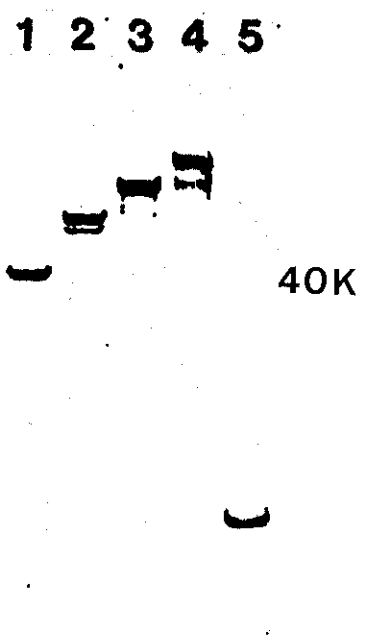

Fig. 2. SDS-PAGE of the conjugates. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gradient gel, 4 to 20%). Gel was then stained and dried. 1, chFabA7; 2, chFabA7-1NCS; 3, chFabA7-2NCS; 4, chFabA7-3NCS; 5, NCS.

expressed as the percentage colony formation compared with the medium control. In the antigen-positive cancer cell line HLC-1, both the conjugate and free NCS inhibited colony formation in a dose-dependent manner

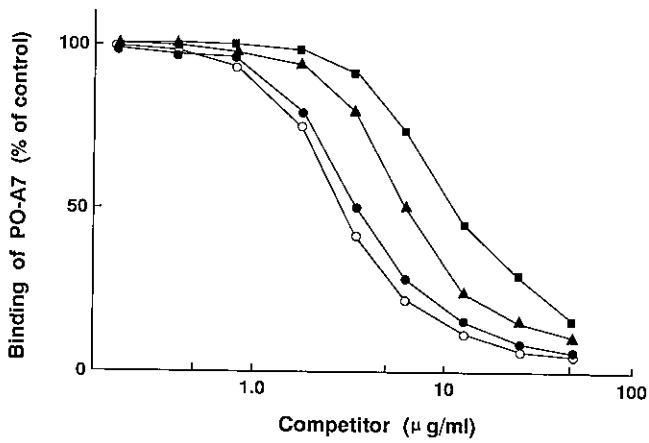

Fig. 3. Binding competition between PO-A7Fab and chFabA7-NCS in HLC-1 cells. chFabA7-1NCS showed identical antigen-binding activity to parent chFabA7 but the antigen-binding activities of chFabA7-2NCS and chFabA7-3NCS were reduced. chFabA7, ○; chFabA7-1NCS, ●; chFabA7-2NCS, ▲; chFabA7-3NCS, □.

and inhibition by the complex was stronger than that of free NCS (Fig. 4A). On the other hand, in the antigen-negative cancer cell line HeLa S3, there was no difference in colony formation rate between the conjugate and free NCS (Fig. 4B). Further, the cytotoxic effect of chA7Fab-1NCS was inhibited by the addition of an excess amount of whole Mab A7 (Fig. 5B), but not by addition of non-specific IgG (Fig. 5A).

DISCUSSION

NCS is known to differ in several respects from other anticancer drugs: 1) NCS is composed of two parts, i.e., the chromophore, which is non-proteinous and biologically active, and the apoprotein, which contributes to the stability of the NCS molecule, but does not affect drug activity[11]; 2) due to the structural character of NCS, the conjugation of other reagents to NCS can occur readily without any loss of biological activity[12]; 3) one mole of NCS is more cytotoxic than one mole of other anticancer drugs, and NCS bound to Mab is highly cytotoxic to targeted cancer cells at low substitution ratios.[13] The above properties are ideal for a drug to be coupled to an antibody. A report by Jung *et al.* has for the first time described the conjugation of NCS to an antibody with SPDP.[14] However, the procedure they employed was a complicated one, as it required the disconnection of active chromophore from apoprotein, apoprotein conjugation to antibody and subsequent reconstitution of the active chromophore to the apoprotein-antibody conjugate to restore the biological activity.

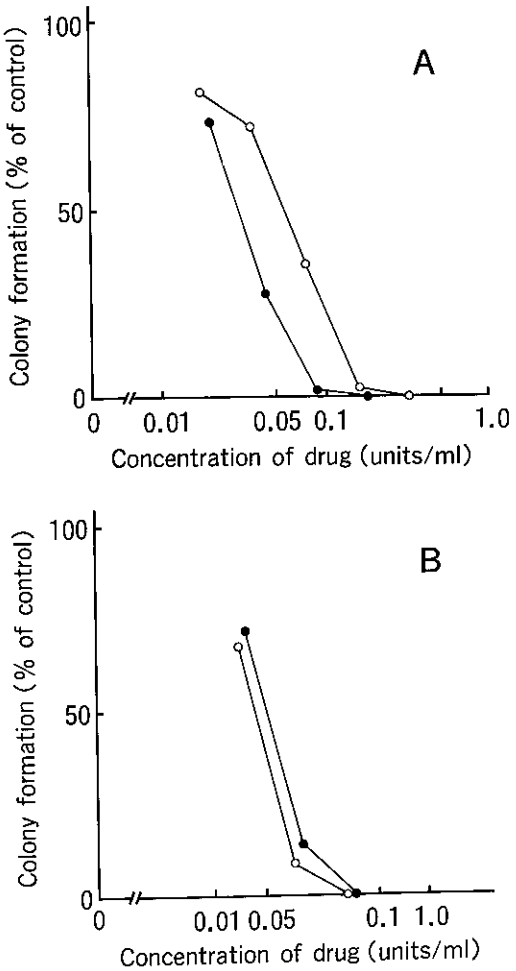

Fig. 4. Cytotoxicity of the conjugate. ChFabA7-1NCS and free NCS were exposed with the antigen-positive HLC-1 cells (A) and the antigen-negative HeLa S3 cells (B). Data were expressed as the percent colony formation as compared with the control. chFabA7-1NCS, ●; free NCS, ○.

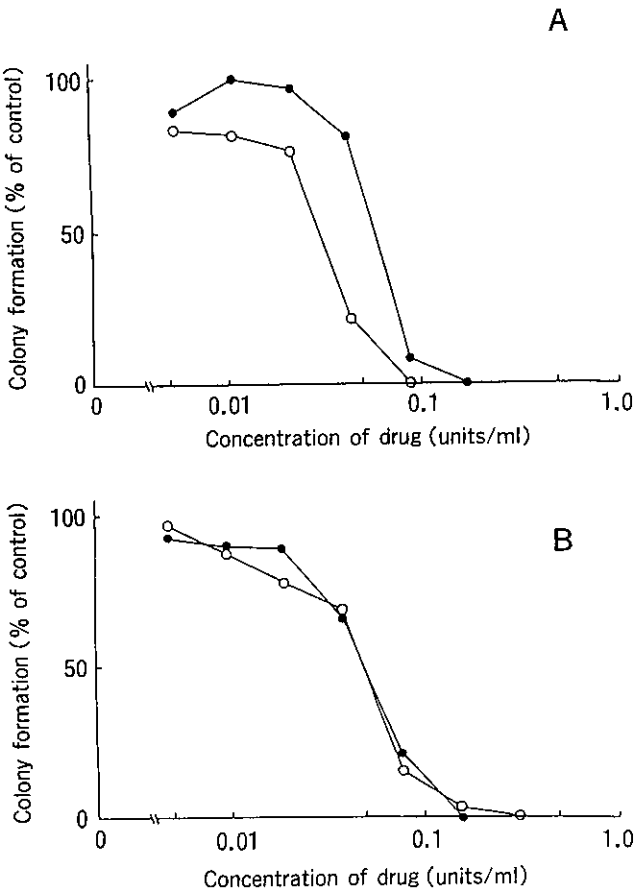

Fig. 5. Effect of an excess amount of non-specific IgG or Mab A7 on the cytotoxicity of the conjugate. The HLC-1 cells were exposed to the conjugate in the presence of non-specific IgG (A) or Mab A7 (B). The colony formation rate of the HLC-1 cells was calculated as compared with the control. NCS, ●; chFabA7-1NCS, ○.

Our procedure, on the other hand, is simpler and does not require chromophore purification or reconstitution with NCS. In addition, our method provides a convenient means of removing free antibodies and thus a conjugate of greater purity can be obtained from the conjugation mixture.

The antigen-binding activity of the conjugates was practically unchanged in chFabA7-1NCS, but markedly reduced in conjugates substituted with two and three moles of NCS. This indicates that the majority of the lysine residues of chFabA7 exposed to NCS conjugation lie within the variable region. Otherwise, steric hinderance by NCS would affect the binding activity. The cytotoxic effect of chFabA7-1NCS on antigen-positive cancer cells was stronger than that of free NCS, chFabA7-2NCS or chFabA7-3NCS, while in antigen-negative cancer cells there was no difference in cytocidal effect between NCS and each conjugate (data not shown). To investigate the antigen specificity of the conjugate, therefore, chFabA7-1NCS was selected for further examination. The cytotoxicity of chFabA7-1NCS was stronger than that of free NCS towards the antigen-positive cells (Fig. 4A), while there was no difference between chFabA7-1NCS and free NCS (Fig. 4B). The cytotoxic effect of chFabA7-1NCS was attenuated by addition of an excess amount of free Mab A7 (Fig. 5). These findings indicate that the cytotoxicity of NCS is enhanced by chFabA7 conjugation and also show that the cytotoxicity of the conjugate is manifested through antigen-antibody interaction.

In conclusion, we have succeeded in the development of a human/chimeric Mab-NCS conjugate without reduction of the antigen-binding activity or cytotoxic effect. chFabA7-1NCS possesses favorable properties and is a promising new tool for targeting colon cancer chemotherapy. This type of conjugate should be superior for clinical application, since it should overcome the problems of human anti mouse antibody (HAMA) production and poor tumor penetration.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan, and by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan.

(Received May 18, 1993/Accepted August 9, 1993)

REFERENCES

1) Miller, R. A. and Levy, R. Response of cutaneous T cell lymphoma to therapy with hybridoma monoclonal antibody. *Lancet*, **ii**, 226–230 (1981).
2) Herlyn, D. and Koplowski, H. IgG_{2a} monoclonal antibodies inhibit human tumor growth through interaction with effector cells. *Proc. Natl. Acad. Sci. USA*, **79**, 4761–4765 (1982).
3) Hurwitz, E. Specific and nonspecific macromolecule-drug conjugates for the improvement of cancer chemotherapy. *Biopolymer*, **22**, 557–567 (1983).
4) Shawler, D. L., Bartholomew, R. M., Smith, L. M. and Dillman, R. O. Human immune response to multiple injection of murine monoclonal IgG. *J. Immunol.*, **135**, 1530–1535 (1985).
5) Tsukada, Y., Ohkawa, K. and Hibi, N. Therapeutic effect of treatment with polyclonal or monoclonal antibodies to α-fetoprotein that have been conjugated to daunomycin via dextran bridge: studies with an α-fetoprotein producing rat hepatoma tumor model. *Cancer Res.*, **47**, 4293–4295 (1987).
6) Takahashi, T., Yamaguchi, T., Kitamura, K., Suzuyama, H., Honda, M., Yokota, T., Kotanagi, H., Takahashi, M. and Hashimoto, Y. Clinical application of monoclonal antibody-drug conjugates for immunotargeting chemotherapy of colorectal carcinoma. *Cancer*, **61**, 881–888 (1988).
7) Takahashi, T., Yamaguchi, T., Kitamura, K., Noguchi, A., Honda, M. and Otsuji, E. Missile therapy for colorectal and pancreatic cancer — clinical trials of monoclonal antibody-drug conjugate A7-NCS. *Jpn. J. Cancer Chemother.*, **17**, 1111–1119 (1990) (in Japanese).
8) Kotanagi, H., Takahashi, T., Masuko, T., Hashimoto, Y. and Koyama, K. A monoclonal antibody against human colon cancers. *Tohoku J. Exp. Med.*, **148**, 353–360 (1986).
9) Kitamura, K., Takahashi, T., Yamaguchi, T., Kitai, S., Amagai, T. and Imanishi, J. Monoclonal antibody A7 tumor localization enhancement by its $F(ab')_2$ fragments to colon carcinoma xenografts in nude mice. *Jpn. J. Clin. Oncol.*, **20**, 139–144 (1990).
10) Kitamura, K., Takahashi, T., Yamaguchi, T., Yokota, T., Noguchi, A., Amagai, T. and Imanishi, J. Immunochemical characterization of the antigen recognized by the murine monoclonal antibody against human colorectal cancer. *Tohoku J. Exp. Med.*, **157**, 83–92 (1989).
11) Napier, M. A., Holmquist, B., Strydam, D. J. and Goldberg, I. H. Neocarzinostatin: spectral characterization and separation of non-protein chromophpre. *Biochem. Biophys. Res. Commun.*, **89**, 635–642 (1979).
12) Koide, Y., Ishii, F., Hasuda, K. and Koyama, Y. Isolation of a protein component from neocarzinostatin (NCS) and their biological activities. *J. Antibiot.*, **33**, 342–346 (1980).
13) Kitamura, K., Takahashi, T., Noguchi, A., Takashina, K., Tsurumi, H. and Yamaguchi, T. Binding, internalization and the cytotoxicity of monoclonal antibody A7-neocarzinostatin conjugates (A7-NCS) in target cells. *Tohoku J. Exp. Med.*, **161**, 199–207 (1990).
14) Jung, G., Kohnlein, W. and Luders, G. Biological activity of the antitumor protein neocarzinostatin coupled to a monoclonal antibody by N-succinimidyl 3-(2-pyridyldithio)propionate. *Biochem. Biophys. Res. Commun.*, **101**, 599–606 (1981).