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Comparative Pharmacokinetic Properties of Murine Monoclonal Antibody A7 Modified with Neocarzinostatin, Dextran and Polyethylene Glycol

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The murine monoclonal antibody A7 (Mab A7) was chemically modified with several macromolecules: dextran, polyethylene glycol and the anti-cancer polypeptide neocarzinostatin. The pharmacokinetic properties of the combinations were subsequently examined. Radioimmunoassay revealed that all preparations retained their antigen-binding activities. The Mab A7-neocarzinostatin conjugate was cleared from the blood circulation with a kinetic pattern almost identical to that of the parent Mab A7. Of the three preparations, Mab A7-dextran (A7-Dx) was removed the most rapidly from the circulation. Mab A7-polyethylene glycol (A7-PEG) exhibited the slowest blood clearance curve, with twice the half life of the parent Mab A7 in the circulation. In normal organ distributions, A7-Dx exhibited the highest liver, spleen and kidney uptake, and A7-PEG showed the lowest uptake, when expressed as tissue:blood ratio. Although A7-Dx exhibited lower tumor uptake, there was no significant difference among the three conjugates in tumor-bearing nude mice. A7-PEG seems to be a good candidate for targeted cancer therapy using antibody due to its high blood retention but low normal organ uptake.

Key words: Monoclonal antibody A7 — Polyethylene glycol — Dextran — Neocarzinostatin — Pharmacokinetics

The development of monoclonal antibody technology has reawakened interest in the use of antibodies as site-specific targeting agents for cancer therapy.[1-4] There are several reports of successful conjugates prepared by coupling a drug directly to an antibody,[5-7] but these types of conjugates probably have a low potential due to a low substitution ratio of drug to antibody. To overcome this deficiency, Takahashi *et al.*[8] have produced the monoclonal antidody A7-neocarzinostatin (A7-NCS) conjugate, with which a sufficient clinical dose can be achieved even with a low substitution ratio of drug to antibody. Since A7-NCS exhibited strong tumoricidal effects in animal experimental models, it was applied clinically. In clinical applications, however, human anti-murine antibody formation was observed in all patients treated with A7-NCS.[9] Therefore, the use of human/mouse chimeric or human monoclonal antibody would be preferable in clinical applications, though no serious adverse side effects were observed following the use of Mab A7. However, these forms of antibody are less stable and more difficult to produce. Another limitation is the smaller amount of antibody obtainable from human-human fused hybridomas or by genetic engineering.

Our study was directed to chemical modification of monoclonal antibody to eliminate anti-murine immune response. Dextran and polyethylene glycol have been used to reduce the antigenicity of proteins.[10-12] In addition, dextran was used as a drug carrier to obtain a higher molar ratio of drug to antibody[10] and polyethylene glycol was used to increase the blood half-lives of proteins, resulting in augmented biological activity.[13] These chemical modifications, however, may alter the pharmacokinetics due to high molecular weight and result in poor tumor localization of the antibody. We prepared the Mab A7-dextran (A7-Dx) conjugate and the Mab A7-polyethylene glycol (A7-PEG) conjugate to evaluate their pharmacokinetics. In the present study, the pharmacokinetics of A7-Dx and A7-PEG were compared with those of the parent Mab A7 and A7-NCS.

MATERIALS AND METHODS

Preparation of murine monoclonal antibody A7 (Mab A7) The Mab A7 was obtained from a hybridoma established by Kotanagi *et al.*[14] Mab A7 was purified from mouse ascitic fluid by protein A affinity chromatography (Bio-Rad, Richmond, CA).[15]

Preparation of A7-NCS Mab A7 was conjugated to NCS via a disulfide bridge as described previously,[8] with a two-to-one molar substitution of NCS to antibody.

Conjugation of dextran to Mab A7 Dextran T70 (Mr: 70 kD) (Pharmacia, Uppsala, Sweden) was conjugated to Mab A7 with a one-to-one molar substitution, as described by Hurwitz *et al.*[16] Briefly, 1 g of dextran T70 was dissolved in 100 ml of 0.03 *M* $NaIO_4$ in 0.1 *M* sodium acetate (pH 5.5), and incubated for 20 h at 4°C. The oxidized dextran was then repeatedly dialyzed against

distilled water, and lyophilized. The oxidized dextran (50 mg) in 2.0 ml of phosphate-buffered saline (PBS) was added to 1 ml of a solution containing 15 mg of purified Mab A7, and the mixture was incubated for 48 h at 4°C. The conjugate was then purified by separation on a Sephadex G 150 column (Pharmacia).

Polyethylene glycol conjugation to Mab A7 Polyethylene glycol (Mr: 5000, Aldrich, WI) was converted to the active ester as described by Abuchowski *et al.*[17] The A7-PEG conjugate was prepared by the following procedure: to 5 mg of Mab A7 in 1 ml of 0.05 *M* PBS (pH 8.0) was added 2.5 mg of activated PEG with a molar ratio of 15, and the mixture was then stirred for 30 min at room temperature. Unbound PEG was removed by gel filtration.

Radiolabeling $Na^{125}I$ was purchased from Amersham International (Amersham, UK). Mab A7, A7-NCS, A7-Dx and A7-PEG were radiolabeled with $Na^{125}I$ by the chloramine T method.[18] The specific activity was 10 μCi/μg of protein in all preparations.

Antigen-binding affinities of the conjugates The antigen-binding activities of the conjugates were assessed using a radioimmunoassay in the presence of hot Mab A7. SW1116, a human colon cancer cell line was used as the target cells throughout this study. Aliquots of SW1116 cells (2×10^5/tube) in RPMI-1640 medium were incubated with 1×10^5 cpm of ^{125}I-labeled Mab A7 in 100 μl of serially diluted Mab A7 or its conjugates. The incubation was performed for 30 min at room temperature in Eppendorf tubes. After centrifugation, the cell pellets were subjected to gamma counting and the percentage inhibition was determined by comparison with a buffer control.

Blood clearance Radiolabeled Mab A7, A7-NCS, A7-Dx, A7-PEG (5×10^6 cpm each) were injected intravenously into three mice for each group. Blood samples were taken from the tail vein at various time points after injection. The samples were then subjected to gamma counting. The data were expressed as a percentage of the initial concentration at 15 min after injection.

Tissue distribution Antibody or conjugate preparations (5×10^6 cpm) were injected intravenously into four mice for each group. The mice were killed 24 h ater injection, and their visceral organs were removed for gamma counting.

Tumor localization Cells from SW1116 were injected subcutaneously into the backs of nude mice (BALB/c, *nu/nu*). The mice developed a solid tumor 1–2 weeks after injection. Rabiolabeled antibody and conjugate preparations (5×10^6 cpm) were then injected intravenously, and the mice were killed 24 h after injection. Excised tumors were sujected to gamma counting, and the data were expressed as a percentage of the injected dose per gram. In a separate experiment, the tumor clearance of A7-PEG was compared with that of the parent Mab A7.

RESULTS

Binding activity The molar ratios of macromolecule to antibody were 1, 2 and 5 for A7-Dx, A7-NCS and A7-PEG, respectively. Figure 1 shows the binding activities of each preparation found by radioimmunoassay. All conjugates showed almost the same binding pattern as the parent Mab A7, although a slight decline in A7-Dx and A7-PEG was observed. The result indicates that all conjugates retained their antigen-binding activity.

Blood clearance In all preparations, the clearance curves showed an initial, rapid decline followed by a much slower decline (Fig. 2). The A7-NCS concentration was reduced with a kinetic pattern nearly identical to that of the parent Mab A7, although there was a rapid decline in the late phase as compared with the parent Mab A7. A7-Dx was removed the fastest from the blood of the four preparations in both the early and late phases. The A7-PEG reduction ratio was the slowest among the four preparations. The blood half-lives for Mab A7 and A7-PEG were 24 h and 48 h, respectively.

Tissue distribution Radiolabeled Mab-A7 or conjugates were given i.v. to four mice for each group. The liver, spleen, kidneys and lungs were removed for gamma counting 24 h after injection, and their radioactivities were compared to that of the blood. Data were expressed as tissue:blood ratio. A7-NCS exhibited an organ uptake nearly identical to that of the parent Mab A7. A7-Dx was highly sequestered in the liver, spleen and kidneys when

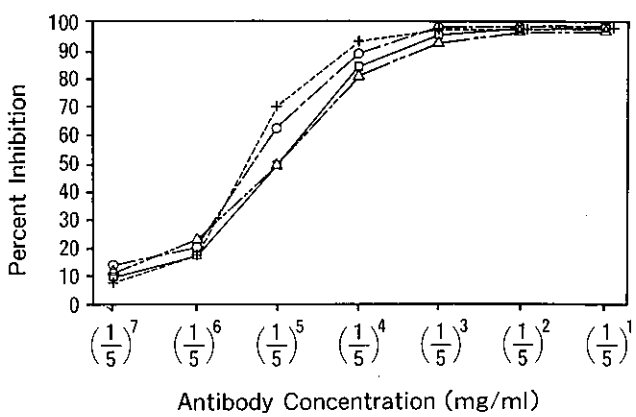

Fig. 1. Binding activity of conjugates. Cells were incubated with 10^5 cpm of ^{125}I-labeled antibody A7, containing various concentrations of conjugates (Mab A7 +, A7-NCS ○, A7-Dx △, A7-PEG □). After washing and centrifugation, the cell pellets were subjected to gamma counting. Data are expressed as percentage inhibition in comparison with control buffer.

compared to the other three preparations. A7-PEG exhibited the lowest accumulation in all organs examined (Fig. 3).

In a separate experiment, the organ accumulation of A7-PEG was compared to that of the parent Mab A7. The results are expressed as the percentage of the injected dose per gram 24 h after injection. A higher percentage of injected dose per gram was found in liver, spleen and lungs for the parent Mab A7 (Table I).

Tumor localization The tumor localizations of three conjugates were expressed as the percent of injected dose per gram of tumor compared to that of the parent Mab A7. As shown in Fig. 4, A7-Dx exhibited a lower tumor uptake when compared with the parent Mab A7, whereas A7-NCS and A7-PEG showed tumor uptakes nearly identical to that of the parent Mab A7.

Comparison of tumor clearance between Mab A7 and A7-PEG To examine the difference in tumor clearance between the parent Mab A7 and A7-PEG, tumor uptakes were examined at 1, 3, 5 and 7 days after administration. Data were expressed as the percent of injected dose per gram of tumor. A7-PEG exhibited a tumor clearance

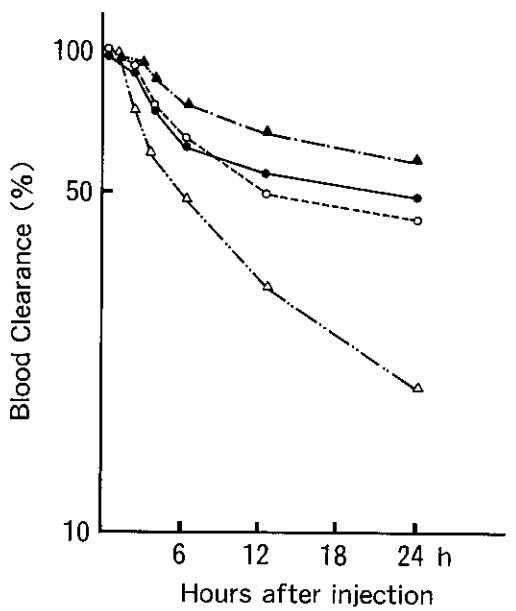

Fig. 2. Blood clearance of conjugates. Radiolabeled conjugates (Mab A7 ●, A7-NCS ○, A7-Dx △, A7-PEG ▲) were intravenously injected into three for each group. Blood samples were taken from the tail vein and subjected to gamma counting. Data are expressed as percentages of the radioactivity in the blood 15 min after injection.

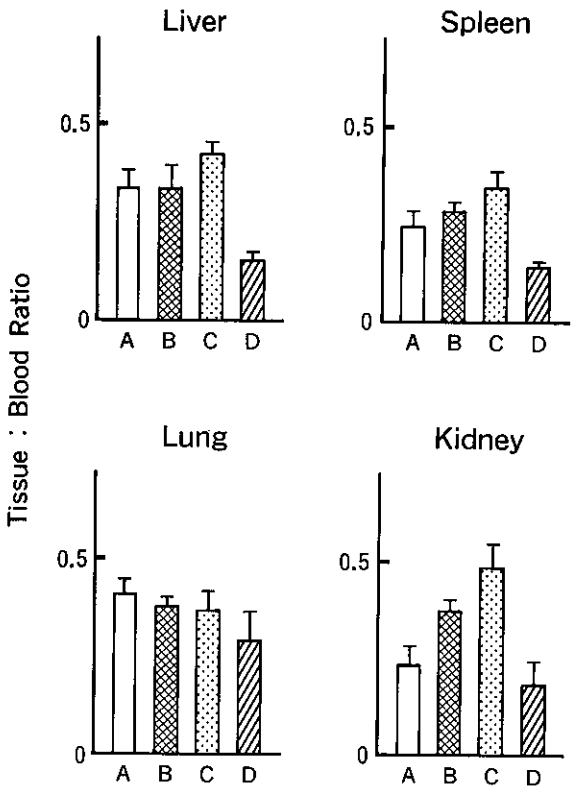

Fig. 3. Tissue distributions. Radiolabeled conjugates (Mab A7; A, A7-NCS; B, A7-Dx; C, A7-PEG; D) were injected intravenously into four mice for each group. Visceral organs were excised 24 h after injection and subjected to gamma counting with serum aliquots. Data are expressed as tissue:blood ratios.

Table I. Comparison of Distribution between Mab A7, A7-NCS, A7-Dx and A7-PEG 24 h after Injection

	Liver	Spleen	Kidney	Lung	Blood
A7	2.48±0.20[a)]	2.02±0.12	1.64±0.15	3.01±0.22	7.49±0.21
A7-NCS	2.37±0.31	2.10±0.16	2.56±0.18	2.70±0.17	6.98±0.23
A7-Dx	1.51±0.17	1.25±0.13	1.73±0.08	1.38±0.11	3.62±0.09
A7-PEG	1.52±0.21	1.49±0.08	1.69±0.01	2.70±0.09	10.06±0.12

a) Data are expressed as a percentage of the injected dose per gram of tissue (mean±SD).

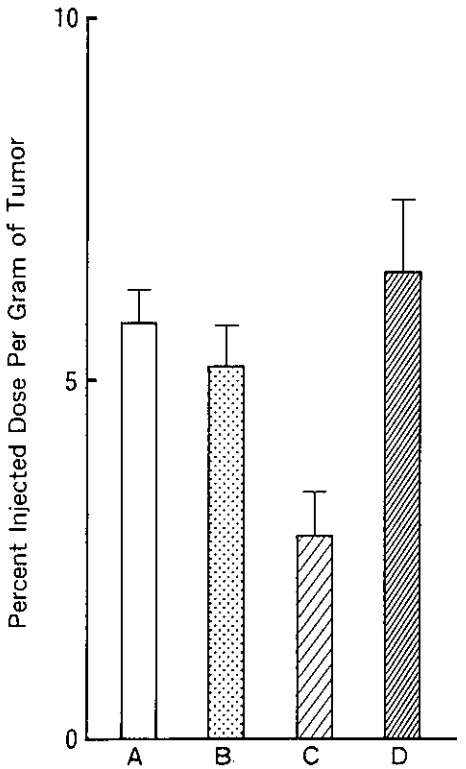

Fig. 4. Tumor localization of conjugates. Radiolabeled conjugates (Mab A7; A, A7-NCS; B, A7-Dx; C, A7-PEG; D) were injected intravenously into four mice for each group. The tumor was removed 24 h after injection and subjected to gamma counting Data are expressed as percentages of the injected per gram of tumor.

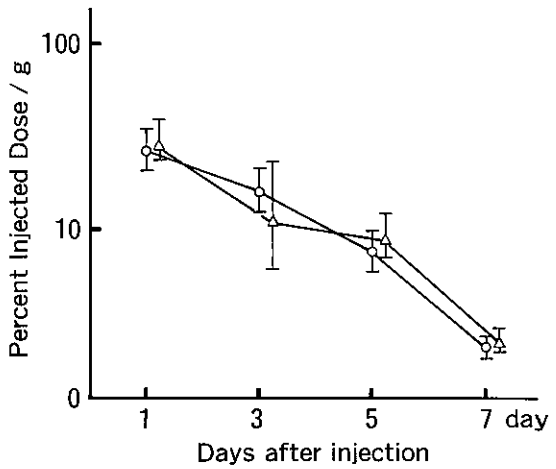

Fig. 5. Tumor clearance of A7-PEG. Radiolabeled A7-PEG (△) or Mab A7 (○) were injected into the tumors of four nude mice. The mice were killed 1, 3, 5 and 7 days after injection. Excised tumors were subjected to gamma counting. Data are expressed as percentages of the injected dose per gram of tissue.

similar to that of the parent Mab A7, except for slightly less uptake 3 days after injection (Fig. 5).

DISCUSSION

In the present study, the pharmacokinetics of chemically modified monoclonal antibody was examined. This involved assessment of the blood, tumor and organ levels of the conjugates in comparison with the levels of the parent Mab A7.

Initially, radioimmunoassay was conducted to confirm that the modified antibodies retained their antigen-binding activities before and after conjugation. These tests showed that all the prepared conjugates retained antigen-binding activities nearly identical to that of the parent Mab A7 by using the human colon cancer cell line, SW1116. Since all conjugates prepared in this report have been developed with low substitution ratios, the antigen-binding sites appeared not to have been destroyed by the conjugation process.

The blood level of A7-NCS declined with a profile similar to, but not precisely paralleling, that of the parent Mab A7. Dx modification reduced the blood retention of the antibody, while PEG modification prolonged the blood half life of the antibody. In preliminary experiments, there was no observed effect on antibody blood clearance by simple addition of Dx, NCS or PEG to antibody solution. Covalent binding of dextran or PEG to the parent Mab A7, therefore, altered the blood half-life of the antibody. In all preparations, clearance curves showed an initial rapid decline followed by a much slower decline. The precise analysis of the clearance curves may require mathematical or computerized analysis. The faster clearance of A7-Dx may suggest that Dx conjugation augments the visceral organ distribution of Mab A7, especially in the liver, spleen and kidneys. On the other hand, A7-PEG exhibited a slower clearance. This may indicate that A7-PEG is taken up by the normal organs to a lesser extent, and that it is less susceptible to proteolysis and glomerular filtration than the parent Mab A7.

To confirm the differences in normal organ distribution of the four preparations, visceral organ uptakes were measured with respect to the tissue:blood ratio. A7-NCS assumed a distribution pattern nearly identical to that of the parent Mab A7. A7-Dx showed higher uptake in the liver, spleen and kidneys. A7-PEG was shown to possess the lowest tissue:blood ratios in all organs examined, when compared to the parent Mab A7 and the other

conjugates. The low tissue:blood ratio observed for A7-PEG can be partly explained by a high retention of A7-PEG in the circulation. Another possibility is an inherent molecular property that restricts uptake of A7-PEG into the organs: PEG may sterically hinder the Fc portion of Mab A7, which is related to non-specific binding.

Tumor levels of the four preparations, expressed as percent of injected dose per gram of tumor, did not show any prominent differences, except for a lowered uptake of A7-Dx. The lowered tumor uptake of A7-Dx could be explained by the observation that A7-Dx had a lower blood retention and higher normal organ distribution. Tumor uptake of A7-PEG was not very high in spite of a high blood retention and a low non-specific uptake. The reason why a lower-than-expected tumor uptake for A7-PEG is observed may be a blockade of transcapillary filtration due to high molecular weight. PEG-modified proteins are believed to have greater molecular weights than predicted by calculation of the PEG number attached to proteins. Tumor clearance of A7-PEG was compared with that of Mab A7, and the area under the concentration curve (AUC) was used to show the total amount of reagent accumulated in the tumor. There was no observed difference in AUC between Mab A7 and A7-PEG (data not shown). The less non-specific uptake of antibody into the normal organs, however, may also imply that PEG increases the actual amount of Mab A7 reaching the target tumor through a specific antigen antibody interaction.

The possibility must be considered that PEG modification will result in difficulty in linking a drug to the antibody. Recently, however, we succeeded in the production of the A7-PEG adriamycin (ADR) conjugate, which contains 10 to 20 molecules of ADR. The A7-PEG-ADR conjugate retains antigen-binding activity and strong cytotoxicity to the target cells *in vitro*. We are now attempting to produce A7-PEG-NCS conjugate. PEG should be conjugated to A7-NCS without difficulty, since our activated PEG can be attached to the antibody or protein portion of NCS simply by mixing. The details of these new types of drug conjugates will be presented in the near future.

In summary, the present study evaluated the radiodistributions of monoclonal antibody A7 chemically modified with NCS, Dx and PEG. A7-NCS had nearly identical pharmacokinetic properties to the parent Mab A7. A7-PEG and A7-Dx showed favorable and unfavorable tumor-targeting specificities from the viewpoint of pharmacokinetics, respectively. These findings indicate that the PEG-modified Mab A7 has potential as a carrier molecule in tumor-targeting chemotherapy.

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