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Importance of the Antigen-Binding Valency and the Nature of the Cross-Linking Bond in Ricin A-Chain Conjugates with Antibody

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As a continuation of our work on toxin A-chain conjugates with antitumor antibodies for selective delivery of the toxin to the target cells, four ricin A-chain conjugates were prepared by linking A-chain to Fab′ or $F(ab')_2$ of rabbit IgG against L1210 with or without employing a cross-linking agent, *N,N′-o*-phenylenedimaleimide (PDM), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or *N*-succinimidyl *m*-(*N*-maleimido)benzoate (SMB), and the effects of antigen-binding valency and of the nature of the cross-linking bond on their *in vitro* cytotoxicity were studied. The relative potencies of the conjugates in terms of IC_{50}'s were as follows: $F(ab')_2$–SPDP–A-chain, 100; Fab′–S–S–A-chain, 21; $F(ab')_2$–SMB–A-chain, 1.3; Fab′–PDM–A-chain 0.38. Among the four conjugates, $F(ab')_2$–SPDP–A-chain and Fab′–S–S–A-chain can be cleaved into the homing and the cytotoxic components with 2 mM 2-mercaptoethanol. These results suggest that divalency in antigen-binding and susceptibility of the cross-linking bond to cleavage by mercapto reagent are desirable for high potency. Protein synthesis in a cell-free system of rabbit reticulocyte lysate was inhibited by Fab′–S–S–A-chain and by Fab′–PDM–A-chain as effectively as by free A-chain, indicating that the liberation of A-chain is not important, at least on ribosomes, but it is important for the A-chain to reach a ribosome after binding of the conjugates to the cell-surface.

Since most of the present cytotoxic antitumor agents show unwanted side-effects due to their detrimental action on normal cells, it is highly desirable in cancer chemotherapy to develop antitumor agents with improved selectivity. As an approach to such agents there has been a considerable amount of research on conjugates of cytotoxic agents with antitumor antibodies (*1*). The importance of this type of approach has been increased owing to the recent rapid advancement of the hybridoma method as a means of preparing antibodies (*2*).

Previously we prepared a conjugate by coupling A-chain of ricin, the intracellularly active enzymic portion of the plant toxin, with the Fab′ fragment of immunoglobulin. The conjugate thus prepared exhibited a potent *in vitro* cytotoxicity

Abbreviations: 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetate; PDM, *N,N′-o*-phenylenedimaleimide; PBS, 10 mM phosphate buffer–0.14 M NaCl, pH 7.2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SMB, *N*-succinimidyl *m*-(*N*-maleimido)benzoate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

against the target cells through its binding to the cell-surface antigens *via* the Fab′ portion of the conjugate (*3*). Several other research groups have also studied the ricin A-chain conjugates with several antibodies including monoclonal antibodies prepared by the hybridoma method (*4–7*). The cytotoxicity of these conjugates against the target cells, however, are in general much smaller than that of ricin. It is extremely desirable to make a more potent antitumor conjugate because of the general paucity of the target antigens on the tumor cell surface. Therefore, in the present study, we have prepared four types of ricin A-chain conjugates with antibody having different linkages for combining the two components of the conjugate and different antigen-binding valencies, and we examined the effect of these variables on the cytotoxic activity.

MATERIALS AND METHODS

Materials—*Ricinus communis* beans were purchased from Kinokuniya Pharmacy, Tokyo; *N,N′-o*-phenylenedimaleimide (PDM) from Nakarai Chemicals, Ltd. Kyoto; *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) from Pharmacia Fine Chemicals AB, Uppsala, Sweden; 2-mercaptoethanol (2-ME) from Nakarai Chemicals, Ltd.; culture medium RPMI1640 from Nissui Seiyaku Co., Tokyo; kanamycin sulfate from Banyu Pharmaceutical Co., Tokyo; fetal calf serum from Grand Island Biological Co., Grand Island, N.Y.; ATP, GTP, creatine phosphate, and creatine kinase from Boehringer Mannheim GmbH, Mannheim; hemin from Sigma Chemical Co., Saint Louis, Mo.; and L-[4,5-^{3}H]leucine (60 Ci/mmol) from the Radiochemical Centre, Amersham. *N*-Succinimidyl *m*-(*N*-maleimido)benzoate (SMB) was synthesized by employing a published method (*8*) with some modification.

L1210 Cells—Murine leukemia L1210 cells were kindly provided by Dr. T. Kataoka, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, and maintained serially by intraperitoneal passage of 1×10^5 cells in a DBA/2 mouse (purchased from Charles River Japan, Atsugi) at weekly intervals. For the cytotoxicity tests, five days after inoculation, ascites L1210 cells were collected in 10 mM phosphate buffer–0.14 M NaCl, pH 7.2 (PBS) and washed three times with the culture medium before use.

The $F(ab')_2$ and Fab′ Fragments and Ricin A-Chain—These components of the conjugates were prepared as previously described (*3*). Briefly, antiserum against L1210 cells was produced in rabbits by four weekly subcutaneous injections of 5×10^8 cells emulsified in complete Freund's adjuvant. The $F(ab')_2$ fragment obtained by pepsin digestion of the IgG fraction of the antiserum was reduced with the minimal amount (2 mM) of 2-ME to give Fab′ resulting from the cleavage of only the inter-heavy-chain disulfide bond. Ricin was extracted from *Ricinus communis* beans, and its A-chain was isolated after treatment with 2-ME (*9*).

The Fab′–S–S–A-Chain Conjugate—This type of conjugate was prepared as previously described (*3*) by the activation of the sulfhydryl group of Fab′–SH with Ellman's reagent followed by the reaction of ricin A-chain with Fab′ having the 3-carboxy-4-nitrophenylthio group introduced.

The Fab′–PDM–A-Chain Conjugate—A mixture of 2.7 mg of PDM and 5 ml of 5 mM Na acetate buffer–0.14 M NaCl–1 mM ethylenediaminetetraacetate (EDTA), pH 5.5 (buffer A) was vigorously stirred for 5 min and centrifuged. A 1.75 ml aliquot of the supernatant (saturated PDM solution) was added to 1.75 ml of Fab′–SH in buffer A (3.9 mg protein/ml), and the reaction was allowed to proceed at room temperature for 30 min. The excess PDM was removed by gel filtration on Sephadex G-25 (0.8×43 cm) in buffer A to give Fab′–PDM. To 2.3 ml of a Fab′-PDM solution (2.1 mg protein/ml) thus obtained were added 0.7 ml of ricin A-chain in buffer A (7.0 mg protein/ml) and 0.3 ml of 0.3 M Na phosphate buffer, pH 6.5, and the reaction was allowed to proceed at 4°C overnight. The mixture was chromatographed on Sephadex G-150 superfine (1.6×93 cm) in 0.9% NaCl to give the pure conjugate Fab′–PDM–A-chain.

The $F(ab')_2$–SPDP–A-Chain Conjugate—Fifty μl of 12 mM SPDP (*10*) in ethanol was added to 2.0 ml of $F(ab')_2$ in 0.1 M Na phosphate buffer–0.1 M NaCl, pH 7.5 (5.0 mg protein/ml), and the mixture was incubated at 26°C for 30 min. The excess SPDP and *N*-hydroxysuccinimide generated were removed by gel filtration on Sephadex G-25 (0.8×43 cm) in buffer A to give $F(ab')_2$–SPDP. The average number of 3-(2-pyridyldithio)propionyl groups introduced into one molecule of $F(ab')_2$ was determined to be 3.0 by reducing the product with

2-ME, followed by measurement of the absorbance at 343 nm due to free pyridine-2-thione generated (*11*). A mixture of 1.9 ml of $F(ab')_2$–SPDP in buffer A (3.1 mg protein/ml), 2.3 ml of ricin A-chain in buffer A (1.8 mg protein/ml), and 0.41 ml of 0.4 M Na phosphate buffer–10 mM EDTA, pH 7.5 was incubated at 26°C for 4 h and chromatographed on Sephadex G-150 superfine (1.6 × 93 cm) in 0.9% NaCl to give the desired conjugate.

The $F(ab')_2$–SMB–A-Chain Conjugate—To 1.0 ml of $F(ab')_2$ in 0.1 M Na phosphate buffer, pH 7.0 (5.2 mg protein/ml) was added 50 μl of a solution of SMB in *N,N*-dimethylformamide (7.0 mg/ml), and the mixture was incubated at 26°C for 30 min. The excess SMB and *N*-hydroxysuccinimide generated were removed by gel filtration on Sephadex G-25 (0.8 × 43 cm) in buffer A to give $F(ab')_2$–SMB. Two ml of $F(ab')_2$–SMB in buffer A (2.2 mg protein/ml) and 2.3 ml of ricin A-chain in buffer A (1.8 mg protein/ml) were mixed, and the reaction was allowed to proceed at room temperature overnight. After removal of a small amount of precipitate by centrifugation, the mixture was chromatographed on Sephadex G-150 superfine (1.6 × 93 cm) in 0.9% NaCl to give the desired conjugate.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)—The conjugates were analyzed by SDS–PAGE carried out by the method of Weber and Osborn (*12*) before and after reduction with 2-ME. In the latter case, 0.1 ml of a conjugate in 0.9% NaCl (0.5–1.0 mg protein/ml) was treated with 10 μl of 0.3 M Tris-HCl buffer, pH 8.3, containing 20 mM 2-ME at 37°C for 1 h and then with 10 μl of 0.2 M iodoacetamide in water at room temperature for 30 min.

Cytotoxicity Assay—Serially diluted conjugates were added to RPMI1640 medium (0.2 ml) containing L1210 cells (2×10^4/ml), 10% fetal calf serum, 20 μM 2-ME, and kanamycin sulfate (0.1 mg/ml), and after incubation in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h, the number of viable cells was determined by counting undyed cells after the addition of one-tenth volume of 3% trypan blue in PBS. The cytotoxicity of the conjugates is expressed in terms of the molar concentration necessary to decrease the number of viable cells after 48-h incubation from 1.6×10^5/ml (control) to 1×10^4/ml, designated as IC_{94}.

Inhibition of Protein Synthesis in a Cell-Free System by Ricin A-Chain—Rabbit reticulocyte lysate used for the test was prepared according to the method of Pelham and Jackson (*13*) and stored at −80°C. Serially diluted conjugates and other test samples were added to 50 μl of the assay mixture containing the above lysate (20 μl), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (15 mM, pH 7.6), CH_3COOK (80 mM), $Mg(CH_3COO)_2$ (1 mM), ATP (1 mM), GTP (0.4 mM), creatine phosphate (8 mM), creatine kinase from rabbit muscle (0.15 mg/ml), hemin (20 μM), L-[4,5-^{3}H]-leucine (0.25 μCi) and cold amino acid mixture without leucine (3 μM). After incubation at 37°C for 10 min, 40 μl of each assay mixture was placed on a paper disk (Whatman 3 MM, 2.5 cm), and the radioactivity of the fraction insoluble in hot trichloroacetic acid was determined on a liquid scintillation counter (Packard 3255) according to the method of Igarashi *et al.* (*14*).

RESULTS

Preparation of Conjugates—Two conjugates which are both monovalent in antigen-binding valency but differ from each other in the cross-linking bond were prepared (Fig. 1a, b). The ricin A-chain conjugate with Fab' coupled by a disulfide bond, Fab'–S–S–A-chain, was prepared by employing Ellman's reagent for the activation of the sulfhydryl group of Fab'–SH. In order to prepare the conjugate cross-linked by a sulfide bond, Fab' was allowed to react with excess PDM to give Fab'–PDM resulting from the addition of the sulfhydryl group of Fab' to only one of the two maleimide groups of the cross-linking agent. Then, Fab'–PDM was treated with ricin A-chain. Figure 2a shows the elution pattern on Sephadex G-150 superfine chromatography of the resulting reaction mixture. The pure conjugate (Fab'–PDM–A-chain) was prepared efficiently by this method, without formation of the homodimer.

Two other conjugates having the divalent antigen-binding site were prepared employing $F(ab')_2$ as the homing component to be coupled with ricin A-chain with SPDP or SMB. The *N*-hydroxysuccinimide ester group, an active ester, of the coupling agents reacts efficiently with the lysine residue of $F(ab')_2$ to form the amide bond. The sulfhydryl group of ricin A-chain reacts with

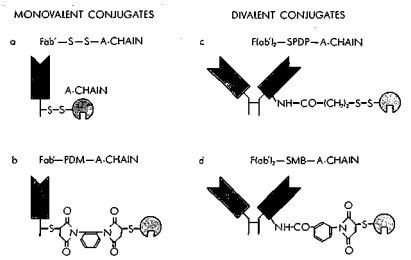

Fig. 1. Structures of ricin A-chain conjugates with antibody. In the cases of the divalent conjugates, they contain $F(ab')_2$–$(A\text{-chain})_n$ ($n=2$ or 3) in addition to the major product $F(ab')_2$–$(A\text{-chain})_1$. In the figure, however, only the structure of $F(ab')_2$–$(A\text{-chain})_n$ is shown as a representative in each case.

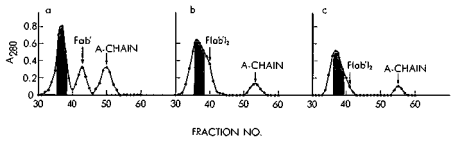

Fig. 2. Elution profile on Sephadex G-150 superfine chromatography of coupling reaction mixtures of Fab′ or $F(ab')_2$ and ricin A-chain. a, Fab′–PDM–A-chain; b, $F(ab')_2$–SPDP–A-chain; c, $F(ab')_2$–SMB–A-chain. Arrows indicate the elution positions of uncoupled components. The fractions indicated by the shaded area were pooled and employed as the conjugates.

the second functional group of the coupling agents remaining in $F(ab')_2$–SPDP (the active disulfide group) and $F(ab')_2$–SMB (the maleimide group) to form the disulfide bond (Fig. 1c) and the sulfide bond (Fig. 1d), respectively. These reaction mixtures gave products whose molecular weights were larger than that of $F(ab')_2$ (Fig. 2b, c). The proteins of the shaded area in Fig. 2a–c were used for the subsequent studies.

Analysis by SDS–PAGE—The molecular weights of the conjugates and the susceptibility of the cross-linking bond to reductive cleavage were determined by SDS–PAGE. The conjugates Fab′–PDM–A-chain and Fab′–S–S–A-chain both migrated to the position of molecular weight 76,000 daltons, which is close to the sum of the molecular weights of Fab′ (46,000 daltons) and A-chain (32,000 daltons), supporting the view that the conjugates were composed of one molecule of Fab′ and one molecule of A-chain (Fig. 3, disks 3 and 4). These conjugates and intact ricin were treated with 2-ME (final 2 and 20 mM). Although Fab′–S–S–A-chain was completely cleaved to Fab′ and A-chain even at the lower (2 mM) concentration of 2-ME (Fig. 3, disk 7), Fab′–PDM–A-chain and ricin remained intact at this concentration

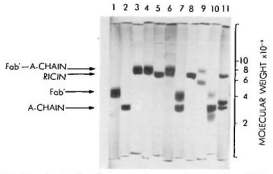

Fig. 3. Analysis of monovalent conjugates by SDS–PAGE. Disk 1, Fab′; disk 2, ricin A-chain; disk 3, Fab′–PDM–A-chain; disk 4, Fab′–S–S–A-chain; disk 5, ricin; disk 6, Fab′–PDM–A-chain treated successively with 2 mM 2-ME and iodoacetamide; disk 7, Fab′–S–S–A-chain treated successively with 2 mM 2-ME and iodoacetamide; disk 8, ricin treated successively with 2 mM 2-ME and iodoacetamide; disk 9, Fab′–PDM–A-chain treated successively with 20 mM 2-ME and iodoacetamide; disk 10, Fab′–S–S–A-chain treated successively with 20 mM 2-ME and iodoacetamide; disk 11, ricin treated successively with 20 mM 2-ME and iodoacetamide.

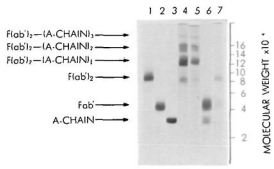

Fig. 4. Analysis of divalent conjugates by SDS–PAGE. Disk 1, $F(ab')_2$; disk 2, Fab′; disk 3, ricin A-chain; disk 4, $F(ab')_2$–SPDP–A-chain; disk 5, $F(ab')_2$–SMB–A-chain; disk 6, $F(ab')_2$–SPDP–A-chain treated successively with 2 mM 2-ME and iodoacetamide; disk 7, $F(ab')_2$–SMB–A-chain treated successively with 2 mM 2-ME and iodoacetamide.

(Fig. 3, disks 6 and 8). Fab′–PDM–A-chain did not give free A-chain even at the higher (20 mM) concentration of 2-ME, though the disulfide bond between the L and H chains was cleaved to give a considerable amount of H-chain fragment–PDM–A-chain (Fig. 3, disk 9). Ricin, at this concentration, was considerably cleaved at its disulfide bond giving rise to free A-chain (Fig. 3, disk 11).

The conjugates $F(ab')_2$–SPDP–A-chain and $F(ab')_2$–SMB–A-chain gave four bands (Fig. 4, disks 4 and 5). Their molecular weights as determined from the respective migration positions suggested that the proteins detected separately were the $F(ab')_2$ molecule having zero, one, two, and three molecules of A-chain; $F(ab')_2$, $F(ab')_2$–$(A\text{-chain})_1$, $F(ab')_2$–$(A\text{-chain})_2$, and $F(ab')_2$–$(A\text{-chain})_3$. The major conjugate produced was $F(ab')_2$–$(A\text{-chain})_1$, whichever of the two coupling agents, SPDP and SMB, was employed. When these conjugates were treated with 2-ME (final 2 mM), $F(ab')_2$–SPDP–A-chain was cleaved to Fab′ and A-chain (Fig. 4, disk 6), but $F(ab')_2$–SMB–A-chain was cleaved to Fab′ and Fab′–SMB–A-chain, and no free A-chain was detected (Fig. 4, disk 7).

Cytotoxicity of the Conjugates—The target cell cytotoxicity was examined by determining the cell viability of L1210 cells cultured with the conjugates. Figure 5a shows the cytotoxicity of the conjugates having the monovalent carrier moiety, Fab′. Although an equimolar mixture of Fab′ and A-chain did not affect the cell growth, Fab′–PDM–A-chain decreased the viability. However, the concentration required to suppress the number of the viable cells to 10^4/ml at the assay time (IC_{94}) was 3.9×10^{-7} M, considerably larger than that (7.2×10^{-9} M) of Fab′–S–S–A-chain. Therefore, for the manifestation of cytotoxicity, the disulfide bond is better than the sulfide bond for coupling the homing and toxic components of the conjugates. The IC_{94} of ricin was found to be 3.3×10^{-11} M, and the cytotoxicity of Fab′–S–S–A-chain was 1/200 of the highly potent cytotoxicity of ricin.

Next, the cytotoxicities of the divalent conjugates were compared with each other (Fig. 5b). The IC_{94}'s of $F(ab')_2$–SPDP–A-chain, $F(ab')_2$–SMB–A-chain, and an equimolar mixture of $F(ab')_2$ and A-chain were 1.5×10^{-9}, 1.2×10^{-7}, and $> 10^{-6}$ M, respectively. Here again, the SPDP conjugate from which A-chain could be reductively liberated with 2-ME exhibited a greater cytotoxicity than the SMB conjugate. The divalent conjugates $F(ab')_2$–SPDP–A-chain and $F(ab')_2$–SMB–A-chain were five and three times more cytotoxic than the corresponding monovalent conjugates Fab′–S–S–A-chain and Fab′–PDM–A-chain, respectively.

Protein Synthesis Inhibitory Activity in a Cell-Free System—Fab′–S–S–A-chain and Fab′–PDM–

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